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(54) Title: **METHOD**

(57) Abstract: A method of producing a replication defective retrovirus comprising transfecting a producer cell with the following: iii) a retroviral genome; iv) a nucleotide sequence coding for retroviral gag and pol proteins; and iii) nucleotide sequences encoding other essential viral packaging components not encoded by the nucleotide sequence of (ii); characterised in that the nucleotide sequence coding for retroviral gag and pol proteins is codon optimised for expression in the producer cell.

Method

Field of the Invention

The present invention relates to methods of improving the safety of retroviral
5 vectors capable of delivering therapeutic genes for use in gene therapy, and to
novel nucleotide sequences for use in such methods.

Background to the Invention

10 Retroviral vectors are now widely used as vehicles to deliver genes into cells.
Their popularity stems from the fact that they are easy to produce and mediate
stable integration of the gene that they carry into the genome of the target cell.
This enables long-term expression of the delivered gene (1).

15 There has been considerable interest, for some time, in the development of retroviral
vector systems based on lentiviruses. Lentiviruses are a small subgroup of complex
retroviruses. They contain, in addition to the common retroviral genes (*gag*, *pol* and
env), genes which enable them to regulate their life cycle and to infect non-dividing
cells (2). Vector systems based thereon are therefore of interest because of their
20 potential use in the transfer of a gene of interest to non-dividing cells such as
neurones. In addition, lentiviral vectors enable very stable long-term expression of
the gene of interest. This has been shown to be at least three months for
transduced rat neuronal cells while MLV based vectors were only able to express
the gene of interest for six weeks.

25 The most commonly used lentivirus is the Human Immunodeficiency Virus (HIV),
the etiologic agent of AIDS (acquired immune deficiency syndrome). HIV-based
vectors have been shown to efficiently transduce non-dividing cells (3) and can be
used, for example, to target anti-HIV therapeutic genes to HIV susceptible cells.

30

However, HIV vectors have a number of significant disadvantages that may limit their therapeutic application to certain diseases. In particular, HIV-1 is a human pathogen carrying potentially oncogenic proteins and sequences. There is the risk that introduction of vector particles produced in packaging cells which express
5 HIV gag-pol will introduce these proteins into the patient leading to seroconversion.

Emphasis has therefore been placed on the safety of these vectors. One strategy looks at the design of production systems for retroviral vectors. A retrovirus
10 vector system basically consists of two elements, a packaging cell line and a vector genome. The simplest packaging line consists of a provirus in which the ψ sequence (a determinant of RNA packaging reporting in HIV as lying between U5 and gag) has been deleted. When stably transfected into a cell, virus particles containing reverse transcriptase will be produced but virion RNA will not
15 become packaged within these particles. The complementing component in a retrovirus vector system is the genome vector itself. The genome vector needs to contain a packaging sequence but much of the structural coding regions can be deleted. Often a selectable marker gene, or other nucleotide sequence of interest, is incorporated into the vector. Vector stocks of the packaging line can then be
20 used to infect target cells. Provided the cell is successfully infected by the viral particle, the genome vector sequence will be reverse transcribed and integrated by the retroviral machinery. However, infection is an end process so no further replication or spread of the vector should occur.

25 As indicated above, however, problems are encountered in the design of safe and effective retroviral vectors. These include the possibility that recombination between the packaging vector and the packaging sequence can lead to the generation of wild type replication competent virus. Consequently efforts have been directed at improving the safety of packaging cell constructs.

30

In second generation packaging cell lines, in addition to deletion of the packaging sequence, the 3' LTR was also deleted so that two recombinations are necessary to generate a wild type virus.

- 5 In third generation packaging lines the *gag-pol* genes and *env* gene are placed on separate constructs that are sequentially introduced into the packaging cells to prevent recombination during transfection.

- With regard to the packaging signal, EP 0 368 882A (Sodroski) discloses that in
10 HIV it corresponds to the region between the 5' major splice donor and the *gag* initiation codon, and particularly corresponds to a segment just downstream of the 5' major splice donor, and about 14 bases upstream of the *gag* initiation codon. It is this region which Sodroski teaches should be deleted from the *gag-pol* cassette. WO97/12622 (Verma) describes that in HIV-1 a 39 bp internal
15 deletion in the ψ sequence can be made between the 5' splice donor site and the starting codon of the *gag* gene.

- Codon wobbling can be used to reduce recombination frequency while maintaining the primary protein sequence of the constructs, c.f. (4) in which the
20 region of overlap between the *gag-pol* and *env* expression constructs was reduced to 61 bp extending over the common region between *pol* and *env* which are in different reading frames. Transversion mutations were introduced into the final 20 codons of *pol*, retaining the integrity of the coding region while reducing the homology with *env* to 55% in the overlap region. Similarly wobble mutations
25 were introduced into the 3' of *env* and all sequences downstream of the *env* stop codon were deleted.

Efficient vectors usually contain part of *gag* on the genome vector to increase virion titre. Unlike the packaging sequence which can be in any position within a

sequence to effect packaging, the *gag* sequence must be in its native position adjacent to ψ to have any effect.

5 It will be appreciated that whilst significant improvements in packaging cell and vector design have been made there is still scope for further refinement of current packaging lines.

Summary of the Invention

10 It is therefore an aim of the invention to provide retroviral particles, in particular lentiviral particles, and particularly those which carry nucleotide constructs encoding therapeutic proteins, that have improved safety over the corresponding wild type viral particle. In our WO99/41397 we describe codon optimisation of the *gag-pol* genes as a means of overcoming the Rev/RRE requirement for export
15 and to enhance RNA stability. We have now found however that the codon optimised *gag-pol* sequence overcomes potential recombination problems with vector genomes which carry part of a *gag* sequence with the aim of increasing titre. This strategy also avoids the need to use *gag* regions from different viruses in the packaging and vector genome constructs.

20 Another significant advantage provided by the invention is that the codon optimisation disrupts RNA secondary structures, such as the packaging signal, thus rendering the *gag-pol* mRNA non-packagable. Thus, the present invention allows retroviral sequence upstream of the *gag* initiation codon to be retained, in contrast to Sodroski and Verma, without significantly compromising safety.

25

Statements of the Invention

Accordingly in one aspect the present invention provides use of a nucleotide sequence coding for retroviral gag and pol proteins, capable of assembly of a retroviral vector genome into a retroviral particle in a producer cell, to generate a

replication defective retrovirus in a target cell, wherein the nucleotide sequence is codon optimised for expression in the producer cell.

Thus in one embodiment the present invention provides use of a nucleotide sequence coding for retroviral gag and pol proteins capable of assembly of a retroviral vector genome into a retroviral particle in a producer cell to reduce or prevent packaging of the retroviral vector genome in a target cell, wherein the nucleotide sequence is codon optimised for expression in the producer cell.

In another embodiment the present invention provides use of a nucleotide sequence coding for retroviral gag and pol proteins, capable of assembly of a retroviral vector genome comprising at least part of a *gag* nucleotide sequence into a retroviral particle in a producer cell, to reduce or prevent recombination between said nucleotide sequence coding for retroviral *gag* and *pol* proteins and the at least part of a *gag* nucleotide sequence, wherein the nucleotide sequence coding for retroviral gag and pol proteins is codon optimised for expression in the producer cell.

Put another way the present invention provides a method of producing a replication defective retrovirus comprising transfecting a producer cell with the following:

- i) a retroviral genome;
- ii) a nucleotide sequence coding for retroviral gag and pol proteins; and
- iii) nucleotide sequences encoding other essential viral packaging components not encoded by the nucleotide sequence of (ii);

characterised in that the nucleotide sequence coding for retroviral gag and pol proteins is codon optimised for expression in the producer cell.

Thus in one embodiment the present invention provides a method of reducing or preventing packaging of a retroviral genome in a target cell comprising the steps of:

a. transfecting a producer cell with the following to produce retroviral particles:

- i) a retroviral genome;
- ii) a nucleotide sequence coding for retroviral gag and pol proteins;

and

- iii) nucleotide sequences encoding other essential viral packaging components not encoded by one or more of the nucleotide sequences of (ii); and

b. transfecting a target cell with retroviral particles of step (a); characterised in that the nucleotide sequence coding for retroviral gag and pol proteins is codon optimised for expression in the producer cell.

In another embodiment the present invention provides a method to reduce or prevent recombination between a retroviral vector genome and a nucleotide sequence encoding a viral polypeptide required for the assembly of the viral genome into retroviral particles comprising transfecting a producer cell with the following:

- (i) a retroviral genome comprising at least part of a gag nucleotide sequence;

- (ii) a nucleotide sequence coding for retroviral gag and pol proteins;

and

- (iii) nucleotide sequences encoding other essential viral packaging components not encoded by the nucleotide sequence of (ii);

characterised in that the nucleotide sequence coding for retroviral gag and pol proteins is codon optimised for expression in the producer cell.

We also provide novel codon optimised sequences as shown in SEQ ID NOS: 15
5 and 16 and which may be used in the present invention. However, it will be appreciated that any convenient codon optimised *gag-pol* sequence may be employed in the invention.

The present invention further provides a retroviral particle produced using the sequences of the present invention, and production methods for so doing.

- 5 The present invention also provides a pharmaceutical composition comprising a viral particle according to the present invention, together with a pharmaceutically acceptable diluent or carrier.

By "reducing" we mean that the chance of an event occurring is reduced
10 compared to a comparable population havingg the wild-type *gag-pol* sequence. Within a population the chance of an event occurring may be prevented for an individual retrovirus vector or particle.

Detailed Description of the Invention

15

Various preferred features and embodiments of the present invention will now be described by way of non-limiting example.

The present invention employs the concept of codon optimisation.

20

Codon optimisation has previously been described in our WO99/41397 as a means of overcoming the Rev/RRE requirement for export and to enhance RNA stability. The alterations to the coding sequences for the viral components improve the sequences for codon usage in the mammalian cells or other cells
25 which are to act as the producer cells for retroviral vector particle production. This improvement in codon usage is referred to as "codon optimisation". Many viruses, including HIV and other lentiviruses, use a large number of rare codons and by changing these to correspond to commonly used mammalian codons, increased expression of the packaging components in mammalian producer cells

can be achieved. Codon usage tables are known in the art for mammalian cells, as well as for a variety of other organisms.

By virtue of alterations in their sequences, the nucleotide sequences encoding the packaging components of the viral particles required for assembly of viral particles in the producer cells/packaging cells have RNA instability sequences (INS) eliminated from them. At the same time, the amino acid coding sequence for the packaging components is retained so that the viral components encoded by the sequences remain the same, or at least sufficiently similar that the function of the packaging components is not compromised.

The term "viral polypeptide required for the assembly of viral particles" means a polypeptide normally encoded by the viral genome to be packaged into viral particles, in the absence of which the viral genome cannot be packaged. For example, in the context of retroviruses such polypeptides would include *gag-pol* and *env*. The term "packaging component" is also included within this definition.

As discussed in our WO99/32646, the sequence requirements for packaging HIV vector genomes are complex. The HIV-1 packaging signal encompasses the splice donor site and contains a portion of the 5'-untranslated region of the *gag* gene, which has a putative secondary structure containing 4 short stem-loops. However, additional sequences elsewhere in the genome are also known to be important for efficient encapsidation of HIV. For example, the first 350 bps of the *gag* protein coding sequence may contribute to efficient packaging. Thus, for construction of HIV-1 vectors capable of expressing heterologous genes, a packaging signal extending to 350 bps of the *gag* protein-coding region has been used on the vector genome. We have now found that codon optimisation of the *gag* coding region on the packaging vector, at least in the region into which the packaging signal extends, also has the effect of disrupting packaging of the vector

genome. Thus codon optimisation is a novel method of obtaining a replication defective viral particle.

Also as disclosed in WO99/32646, the structure of the packaging signal in equine lentiviruses is different from that of HIV. Instead of a short sequence of 4 stem loops together with a packaging signal extending to 350 bps of the *gag* protein-coding region, we have found that in equine lentiviruses the packaging signal may not extend as far into the *gag* protein-coding region as may have been thought.

In one embodiment only codons relating to the packaging signal are codon optimised. Thus, in one embodiment, codon optimisation extends to at least the first 350 bps of the *gag* protein coding region. In equine lentiviruses, at least, codon optimisation extends to at least nucleotide 300 of the *gag* coding region, more preferably to at least nucleotide 150 of the *gag* coding region. Although not optimal, codon optimisation could extend to, say, only the first 109 nucleotides of the *gag* coding region. It may also be possible for codon optimisation to extend to only the first codon of the *gag* coding region.

However, in a much more preferred and practical embodiment, the sequences are codon optimised in their entirety, with the exception of the sequence encompassing the frameshift site.

The *gag-pol* gene comprises two overlapping reading frames encoding *gag* and *pol* proteins respectively. The expression of both proteins depends on a frameshift during translation. This frameshift occurs as a result of ribosome "slippage" during translation. This slippage is thought to be caused at least in part by ribosome-stalling RNA secondary structures. Such secondary structures exist downstream of the frameshift site in the *gag-pol* gene. For HIV, the region of overlap extends from nucleotide 1222 downstream of the beginning of *gag*

(wherein nucleotide 1 is the A of the *gag* ATG) to the end of *gag* (nt 1503). Consequently, a 281 bp fragment spanning the frameshift site and the overlapping region of the two reading frames is preferably not codon optimised. Retaining this fragment will enable more efficient expression of the *gag-pol* proteins.

For EIAV the beginning of the overlap has been taken to be nt 1262 (where nucleotide 1 is the A of the *gag* ATG). The end of the overlap is at 1461 bp. In order to ensure that the frameshift site and the *gag*, *gag-pol* overlap the wild type sequence has been retained from nt 1156 to 1465. This can be seen in Figure 9b.

Derivations from optimal codon usage may be made, for example, in order to accommodate convenient restriction sites, and conservative amino acid changes may be introduced into the *gag-pol* proteins.

In a highly preferred embodiment, codon optimisation was based on lightly expressed mammalian genes. The third and sometimes the second and third base may be changed. An example of a codon usage table is given in Figure 3b.

Due to the degenerate nature of the Genetic Code, it will be appreciated that numerous *gag-pol* sequences can be achieved by a skilled worker. Also there are many retroviral variants described and which can be used as a starting point for generating a codon optimised *gag-pol* sequence. Lentiviral genomes can be quite variable. For example there are many quasi-species of HIV-1 which are still functional. This is also the case for EIAV. These variants may be used to enhance particular parts of the transduction process. Examples of HIV-1 variants may be found at <http://hiv-web.lanl.gov>. Details of EIAV clones may be found at the NCBI database: <http://www.ncbi.nlm.nih.gov>.

The strategy for codon optimised *gag-pol* sequences can be used in relation to any retrovirus. This would apply to all the lentiviruses, including EIAV, FIV, BIV, CAEV, VMR, SIV, HIV-1 and HIV-2. In addition this method could be used to increase expression of genes from HTLV-1, HTLV-2, HFV, HSRV and
5 human endogenous retroviruses (HERV).

As codon optimisation may result in disruption of RNA secondary structures such as the packaging signal, it will be appreciated that any endogenous packaging signal upstream of the *gag* initiation codon could be retained without
10 compromising safety.

An additional advantage of codon optimising packaging components is that this can increase gene expression. In particular, it can render *gag-pol* expression Rev independent. In order to enable the use of anti-*rev* or RRE factors in the retroviral vector, however, it would be necessary to render the viral vector generation
15 system totally Rev/RRE independent (5). Thus, the genome also needs to be modified. This is achieved by optimising vector genome components. Advantageously, these modifications also lead to the production of a safer system absent of all accessory proteins both in the producer and in the transduced cell,
20 and are described below.

As described above, the packaging components for a retroviral vector include expression products of *gag*, *pol* and *env* genes. In addition, efficient packaging depends on a short sequence of 4 stem loops followed by a partial sequence from
25 *gag* and *env* (the "packaging signal"). Thus, inclusion of a deleted *gag* sequence in the retroviral vector genome (in addition to the full *gag* sequence on the packaging construct) will optimise vector titre. To date efficient packaging has been reported to require from 255 to 360 nucleotides of *gag* in vectors that still retain *env* sequences, or about 40 nucleotides of *gag* in a particular combination
30 of splice donor mutation, *gag* and *env* deletions. We have surprisingly found that

a deletion of up to 360 nucleotides in *gag* leads to an increase in vector titre. Further deletions resulted in lower titres. Additional mutations at the major splice donor site upstream of *gag* were found to disrupt packaging signal secondary structure and therefore lead to decreased vector titre. Thus, preferably,
5 the retroviral vector genome includes a *gag* sequence from which up to 360 nucleotides have been removed.

We therefore allow the preparation of a so-called "minimal" system in which all of the accessory genes may be removed. In HIV these accessory genes are *vpr*,
10 *vif*, *tat*, *nef*, *vpu* and *rev*. Similarly, in other lentiviruses the analogous accessory genes normally present in the lentivirus may be removed. For the avoidance of doubt, however, we would mention that the present invention also extends to systems, particles and vectors in which one or more of these accessory genes is present and in any combination.

15 The term "viral vector" refers to a nucleotide construct comprising a viral genome capable of being transcribed in a host cell, which genome comprises sufficient viral genetic information to allow packaging of the viral RNA genome, in the presence of packaging components, into a viral particle capable of infecting
20 a target cell. Infection of the target cell includes reverse transcription and integration into the target cell genome, where appropriate for particular viruses. The viral vector in use typically carries heterologous coding sequences (nucleotides of interest or "NOIs") which are to be delivered by the vector to the target cell, for example a first nucleotide sequence encoding a ribozyme. By
25 "replication defective" we mean that a viral vector is incapable of independent replication to produce infectious viral particles within the final target cell.

The term "viral vector system" is intended to mean a kit of parts which can be used when combined with other necessary components for viral particle
30 production to produce viral particles in host cells. For example, an NOI may

typically be present in a plasmid vector construct suitable for cloning the NOI into a viral genome vector construct. When combined in a kit with a further nucleotide sequence, which will also typically be present in a separate plasmid vector construct, the resulting combination of plasmid containing the NOI and
5 plasmid containing the further nucleotide sequence comprises the essential elements of the invention. Such a kit may then be used by the skilled person in the production of suitable viral vector genome constructs which when transfected into a host cell together with the plasmid containing the further nucleotide sequence, and optionally nucleic acid constructs encoding other components
10 required for viral assembly, will lead to the production of infectious viral particles.

Alternatively, the further nucleotide sequence may be stably present within a packaging cell line that is included in the kit.

15

The kit may include the other components needed to produce viral particles, such as host cells and other plasmids encoding essential viral polypeptides required for viral assembly. By way of example, the kit may contain (i) a plasmid containing an NOI and (ii) a plasmid containing a further nucleotide sequence encoding a
20 modified retroviral *gag-pol* construct which has been codon optimised for expression in a producer of choice. Optional components would then be (a) a retroviral genome construct with suitable restriction enzyme recognition sites for cloning the NOI into the viral genome, optionally with at least a partial *gag* sequence; (b) a plasmid encoding a VSV-G env protein. Alternatively,
25 nucleotide sequence encoding viral polypeptides required for assembly of viral particles may be provided in the kit as packaging cell lines comprising the nucleotide sequences, for example a VSV-G expressing cell line.

The term "viral vector production system" refers to the viral vector system described above wherein the NOI has already been inserted into a suitable viral vector genome.

- 5 In the present invention, several terms are used interchangeably. Thus, "virion", "virus", "viral particle", "retroviral particle", "retrovirus", and "vector particle" mean virus and virus-like particles that are capable of introducing a nucleic acid into a cell through a viral-like entry mechanism. Such vector particles can, under certain circumstances, mediate the transfer of NOIs into the cells they infect. A
10 retrovirus is capable of reverse transcribing its genetic material into DNA and incorporating this genetic material into a target cell's DNA upon transduction. Such cells are designated herein as "target cells".

- As used herein the term "target cell" simply refers to a cell which the regulated
15 retroviral vector of the present invention, whether native or targeted, is capable of infecting or transducing.

- A lentiviral vector particle according to the invention will be capable of transducing cells which are slowly-dividing, and which non-lentiviruses such as
20 MLV would not be able to efficiently transduce. Slowly-dividing cells divide once in about every three to four days including certain tumour cells. Although tumours contain rapidly dividing cells, some tumour cells especially those in the centre of the tumour, divide infrequently.

- 25 Alternatively the target cell may be a growth-arrested cell capable of undergoing cell division such as a cell in a central portion of a tumour mass or a stem cell such as a haematopoietic stem cell or a CD34-positive cell.

- As a further alternative, the target cell may be a precursor of a differentiated cell
30 such as a monocyte precursor, a CD33-positive cell, or a myeloid precursor.

As a further alternative, the target cell may be a differentiated cell such as a neuron, astrocyte, glial cell, microglial cell, macrophage, monocyte, epithelial cell, endothelial cell, hepatocyte, spermatocyte, spermatid or spermatozoa.

5

Target cells may be transduced either *in vitro* after isolation from a human individual or may be transduced directly *in vivo*.

10 Viral vectors according to the invention are retroviral vectors, in particular lentiviral vectors such as HIV and EIAV vectors. The retroviral vector of the present invention may be derived from or may be derivable from any suitable retrovirus. A large number of different retroviruses have been identified. Examples include: murine leukemia virus (MLV), human immunodeficiency virus (HIV), simian immunodeficiency virus, human T-cell leukemia virus
15 (HTLV). equine infectious anaemia virus (EIAV), mouse mammary tumour virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian
20 erythroblastosis virus (AEV). A detailed list of retroviruses may be found in Coffin *et al.*, 1997, "Retroviruses", Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763.

25 The term "derivable" is used in its normal sense as meaning a nucleotide sequence such as an LTR or a part thereof which need not necessarily be obtained from a vector such as a retroviral vector but instead could be derived therefrom. By way of example, the sequence may be prepared synthetically or by use of recombinant DNA techniques.

Details on the genomic structure of some retroviruses may be found in the art. By way of example, details on HIV and Mo-MLV may be found from the NCBI Genbank (Genome Accession Nos. AF033819 and AF033811, respectively). Details of HIV variants may also be found at <http://hiv-web.lanl.gov>. Details of
5 ELAV variants may be found through <http://www.ncbi.nlm.nih.gov>.

The lentivirus group can be split even further into "primate" and "non-primate". Examples of primate lentiviruses include human immunodeficiency virus (HIV), the causative agent of human auto-immunodeficiency syndrome (AIDS), and
10 simian immunodeficiency virus (SIV). The non-primate lentiviral group includes the prototype "slow virus" visna/maedi virus (VMV), as well as the related caprine arthritis-encephalitis virus (CAEV), equine infectious anaemia virus (EIAV) and the more recently described feline immunodeficiency virus (FIV) and bovine immunodeficiency virus (BIV).

15 The basic structure of a retrovirus genome is a 5' LTR and a 3' LTR, between or within which are located a packaging signal to enable the genome to be packaged, a primer binding site, integration sites to enable integration into a host cell genome and *gag*, *pol* and *env* genes encoding the packaging components - these
20 are polypeptides required for the assembly of viral particles. More complex retroviruses have additional features, such as *rev* and RRE sequences in HIV, which enable the efficient export of RNA transcripts of the integrated provirus from the nucleus to the cytoplasm of an infected target cell.

25 In the provirus, these genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are responsible for proviral integration, and transcription. LTRs also serve as enhancer-promoter sequences and can control the expression of the viral genes. Encapsidation of the retroviral RNAs occurs by virtue of a *psi* sequence, which it has been disclosed in respect of HIV, at least, is
30 located at the 5' end of the viral genome.

The LTRs themselves are identical sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both
5 ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

In a defective retroviral vector genome *gag*, *pol* and *env* may be absent or not
10 functional. The R regions at both ends of the RNA are repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively.

As discussed above, in a typical retroviral vector for use in gene therapy, at least
15 part of one or more of the *gag*, *pol* and *env* protein coding regions essential for replication may be removed from the viral vector. This makes the retroviral vector replication-defective. The removed portions may even be replaced by a nucleotide sequence of interest (NOI), as in the present invention, to generate a virus capable of integrating its genome into a host genome but wherein the
20 modified viral genome is unable to propagate itself due to a lack of structural proteins. When integrated in the host genome, expression of the NOI occurs - resulting in, for example, a therapeutic and/or a diagnostic effect. Thus, the transfer of an NOI into a site of interest is typically achieved by: integrating the NOI into the recombinant viral vector; packaging the modified viral vector into a
25 virion coat; and allowing transduction of a site of interest - such as a targeted cell or a targeted cell population.

A minimal retroviral genome for use in the present invention may therefore
comprise (5') R - U5 - one or more NOIs - U3-R (3'). However, the plasmid
30 vector used to produce the retroviral genome within a host cell/packaging cell

will also include transcriptional regulatory control sequences operably linked to the retroviral genome to direct transcription of the genome in a host cell/packaging cell. These regulatory sequences may be the natural sequences associated with the transcribed retroviral sequence, i.e. the 5' U3 region, or they
5 may be a heterologous promoter such as another viral promoter, for example the CMV promoter.

Some retroviral genomes require additional sequences for efficient virus production. For example, in the case of HIV, *rev* and RRE sequence should be
10 included. However, we have found that the requirement for *rev* and RRE can be reduced or eliminated by codon optimisation. As expression of the codon optimised *gag-pol* is REV independent, RRE can be removed from the *gag-pol* expression cassette, thus removing any potential for recombination with any RRE contained on the vector genome.

15 Once the retroviral vector NOI sequences need to be expressed. In a retrovirus, the promoter is located in the 5' LTR U3 region of the provirus. In retroviral vectors, the promoter driving expression of a therapeutic gene may be the native retroviral promoter in the 5' U3 region, or an alternative promoter engineered
20 into the vector. The alternative promoter may physically replace the 5' U3 promoter native to the retrovirus, or it may be incorporated at a different place within the vector genome such as between the LTRs.

Thus, the NOI will also be operably linked to a transcriptional regulatory control
25 sequence to allow transcription of the first nucleotide sequence to occur in the target cell. The control sequence will typically be active in mammalian cells. The control sequence may, for example, be a viral promoter such as the natural viral promoter or a CMV promoter or it may be a mammalian promoter. It is particularly preferred to use a promoter that is preferentially active in a particular
30 cell type or tissue type in which the virus to be treated primarily infects. Thus, in

one embodiment, a tissue-specific regulatory sequences may be used. The regulatory control sequences driving expression of the one or more first nucleotide sequences may be constitutive or regulated promoters.

- 5 The term "operably linked" denotes a relationship between a regulatory region (typically a promoter element, but may include an enhancer element) and the coding region of a gene, whereby the transcription of the coding region is under the control of the regulatory region.
- 10 As used herein, the term "enhancer" includes a DNA sequence which binds other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

15 In one preferred embodiment of the present invention, the enhancer is an ischaemic like response element (ILRE).

The term "ischaemia like response element" - otherwise written as ILRE - includes an element that is responsive to or is active under conditions of ischaemia or conditions that are like ischaemia or are caused by ischaemia. By way of example, conditions that are like ischaemia or are caused by ischaemia include hypoxia and/or low glucose concentration(s).

20

The term "hypoxia" means a condition under which a particular organ or tissue receives an inadequate supply of oxygen.

25

Ischaemia can be an insufficient supply of blood to a specific organ or tissue. A consequence of decreased blood supply is an inadequate supply of oxygen to the organ or tissue (hypoxia). Prolonged hypoxia may result in injury to the affected organ or tissue.

30

A preferred ILRE is a hypoxia response element (HRE).

In one preferred aspect of the present invention, there is hypoxia or ischaemia regulatable expression of the retroviral vector components. In this regard,
5 hypoxia is a powerful regulator of gene expression in a wide range of different cell types and acts by the induction of the activity of hypoxia-inducible transcription factors such as hypoxia inducible factor-1 (HIF-1; 6), which bind to cognate DNA recognition sites, the hypoxia-responsive elements (HREs) on various gene promoters. Dachs *et al* (7) have used a multimeric form of the HRE
10 from the mouse phosphoglycerate kinase-1 (PGK-1) gene (8) to control expression of both marker and therapeutic genes by human fibrosarcoma cells in response to hypoxia *in vitro* and within solid tumours *in vivo* (7 *ibid*).

Hypoxia response enhancer elements (HREEs) have also been found in
15 association with a number of genes including the erythropoietin (EPO) gene (9; 10). Other HREEs have been isolated from regulatory regions of both the muscle glycolytic enzyme pyruvate kinase (PKM) gene (11), the human muscle-specific β -enolase gene (ENO3; 12) and the endothelin-1 (ET-1) gene (13).

20 Preferably the HRE of the present invention is selected from, for example, the erythropoietin HRE element (HREE1), muscle pyruvate kinase (PKM), HRE element, phosphoglycerate kinase (PGK) HRE, B-enolase (enolase 3; ENO3) HRE element, endothelin-1 (ET-1)HRE element and metallothionein II (MTII) HRE element.

25

Preferably the ILRE is used in combination with a transcriptional regulatory element, such as a promoter, which transcriptional regulatory element is preferably active in one or more selected cell type(s), preferably being only active in one cell type.

30

As outlined above, this combination aspect of the present invention is called a responsive element.

Preferably the responsive element comprises at least the ILRE as herein defined.

5

Non-limiting examples of such a responsive element are presented as OBHRE1 and XiaMac. Another non-limiting example includes the ILRE in use in conjunction with an MLV promoter and/or a tissue restricted ischaemic responsive promoter. These responsive elements are disclosed in WO99/15684.

10

Other examples of suitable tissue restricted promoters/enhancers are those which are highly active in tumour cells such as a promoter/enhancer from a *MUC1* gene, a *CEA* gene or a *5T4* antigen gene. The alpha-fetoprotein (AFP) promoter is also a tumour-specific promoter. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination.

15

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site.

20

The promoter may be located in the retroviral 5' LTR to control the expression of a cDNA encoding an NOI, and/or gag-pol proteins.

25

Preferably the NOI and/or gag-pol proteins are capable of being expressed from the retrovirus genome such as from endogenous retroviral promoters in the long terminal repeat (LTR).

30

Preferably the NOI and/or gag-pol proteins are expressed from a heterologous promoter to which the heterologous gene or sequence, and/or codon optimised *gag-pol* sequence is operably linked.

Alternatively, the promoter may be an internal promoter.

Preferably the NOI is expressed from an internal promoter.

5

Vectors containing internal promoters have also been widely used to express multiple genes. An internal promoter makes it possible to exploit promoter/enhancer combinations other than those found in the viral LTR for driving gene expression. Multiple internal promoters can be included in a retroviral vector and it has proved possible to express at least three different cDNAs each from its own promoter (14). Internal ribosomal entry site (IRES) elements have also been used to allow translation of multiple coding regions from either a single mRNA or from fusion proteins that can then be expressed from an open reading frame.

15

The promoter of the present invention may be constitutively efficient, or may be tissue or temporally restricted in their activity.

Preferably the promoter is a constitutive promoter such as CMV.

20

Preferably the promoters of the present invention are tissue specific. That is, they are capable of driving transcription of a NOI or NOI(s) in one tissue while remaining largely "silent" in other tissue types.

25

The term "tissue specific" means a promoter which is not restricted in activity to a single tissue type but which nevertheless shows selectivity in that they may be active in one group of tissues and less active or silent in another group.

30

The level of expression of an NOI or NOIs under the control of a particular promoter may be modulated by manipulating the promoter region. For example,

different domains within a promoter region may possess different gene regulatory activities. The roles of these different regions are typically assessed using vector constructs having different variants of the promoter with specific regions deleted (that is, deletion analysis). This approach may be used to identify, for example,
5 the smallest region capable of conferring tissue specificity or the smallest region conferring hypoxia sensitivity.

A number of tissue specific promoters, described above, may be particularly advantageous in practising the present invention. In most instances, these
10 promoters may be isolated as convenient restriction digestion fragments suitable for cloning in a selected vector. Alternatively, promoter fragments may be isolated using the polymerase chain reaction. Cloning of the amplified fragments may be facilitated by incorporating restriction sites at the 5' end of the primers.

15 The NOI or NOIs may be under the expression control of an expression regulatory element, such as a promoter and enhancer.

Preferably the ischaemic responsive promoter is a tissue restricted ischaemic responsive promoter.
20

Preferably the tissue restricted ischaemic responsive promoter is a macrophage specific promoter restricted by repression.

Preferably the tissue restricted ischaemic responsive promoter is an endothelium specific promoter.
25

Preferably the regulated retroviral vector of the present invention is an ILRE regulated retroviral vector.

Preferably the regulated retroviral vector of the present invention is an ILRE regulated lentiviral vector.

Preferably the regulated retroviral vector of the present invention is an
5 autoregulated hypoxia responsive lentiviral vector.

Preferably the regulated retroviral vector of the present invention is regulated by glucose concentration.

- 10 For example, the glucose-regulated proteins (grp's) such as grp78 and grp94 are highly conserved proteins known to be induced by glucose deprivation (15). The grp 78 gene is expressed at low levels in most normal healthy tissues under the influence of basal level promoter elements but has at least two critical "stress inducible regulatory elements" upstream of the TATA element (15 *ibid*; 16).
- 15 Attachment to a truncated 632 base pair sequence of the 5' end of the grp78 promoter confers high inducibility to glucose deprivation on reporter genes *in vitro* (16 *ibid*). Furthermore, this promoter sequence in retroviral vectors was capable of driving a high level expression of a reporter gene in tumour cells in murine fibrosarcomas, particularly in central relatively ischaemic/fibrotic sites
- 20 (16 *ibid*).

Preferably the regulated retroviral vector of the present invention is a self-inactivating (SIN) vector.

- 25 By way of example, self-inactivating retroviral vectors have been constructed by deleting the transcriptional enhancers or the enhancers and promoter in the U3 region of the 3' LTR. After a round of vector reverse transcription and integration, these changes are copied into both the 5' and the 3' LTRs producing a transcriptionally inactive provirus (17; 18; 19; 20). However, any promoter(s)
- 30 internal to the LTRs in such vectors will still be transcriptionally active. This

strategy has been employed to eliminate effects of the enhancers and promoters in the viral LTRs on transcription from internally placed genes. Such effects include increased transcription (21) or suppression of transcription (22). This strategy can also be used to eliminate downstream transcription from the 3' LTR into genomic DNA (23). This is of particular concern in human gene therapy where it is of critical importance to prevent the adventitious activation of an endogenous oncogene.

As discussed above, replication-defective retroviral vectors are typically propagated, for example to prepare suitable titres of the retroviral vector for subsequent transduction, by using a combination of a packaging or helper cell line and the recombinant vector. That is to say, that the three packaging proteins can be provided *in trans*.

In general a "packaging cell line" contains one or more of the retroviral *gag*, *pol* and *env* genes. In the present invention it contains codon optimised *gag-pol* genes, and optionally an *env* gene. The packaging cell line produces the proteins required for packaging retroviral DNA but it cannot bring about encapsidation. Conventionally this has been achieved through lack of a *psi* region. However, when a recombinant vector carrying an NOI and a *psi* region is introduced into the packaging cell line, the helper proteins can package the *psi*-positive recombinant vector to produce the recombinant virus stock. This virus stock can be used to transduce cells to introduce the NOI into the genome of the target cells. Conventionally a *psi* packaging signal, called *psi* plus, has been used that contains additional sequences spanning from upstream of the splice donor to downstream of the *gag* start codon (24) since this has been shown to increase viral titres.

The recombinant virus whose genome lacks all genes required to make viral proteins can transduce only once and cannot propagate. These viral vectors which

are only capable of a single round of transduction of target cells are known as replication defective vectors. Hence, the NOI is introduced into the host/target cell genome without the generation of potentially harmful retrovirus. A summary of the available packaging lines is presented in Coffin *et al.*, 1997 (*ibid*).

5

The retroviral packaging cell line is preferably in the form of a transiently transfected cell line. Transient transfections may advantageously be used to measure levels of vector production when vectors are being developed. In this regard, transient transfection avoids the longer time required to generate stable
10 vector-producing cell lines and may also be used if the vector or retroviral packaging components are toxic to cells. Components typically used to generate retroviral vectors include a plasmid encoding the gag-pol proteins, a plasmid encoding the env protein and a plasmid containing an NOI. Vector production involves transient transfection of one or more of these components into cells
15 containing the other required components. If the vector encodes toxic genes or genes that interfere with the replication of the host cell, such as inhibitors of the cell cycle or genes that induce apoptosis, it may be difficult to generate stable vector-producing cell lines, but transient transfection can be used to produce the vector before the cells die. Also, cell lines have been developed using transient
20 transfection that produce vector titre levels that are comparable to the levels obtained from stable vector-producing cell lines (25).

Producer cells/packaging cells can be of any suitable cell type. Producer cells are generally mammalian cells but can be, for example, insect cells. A producer cell
25 may be a packaging cell containing the virus structural genes, normally integrated into its genome into which the regulated retroviral vectors of the present invention are introduced. Alternatively the producer cell may be transfected with nucleic acid sequences encoding structural components, such as codon optimised gag-pol and env on one or more vectors such as plasmids, adenovirus vectors,
30 herpes viral vectors or any method known to deliver functional DNA into target

cells. The vectors according to the present invention are then introduced into the packaging cell by the methods of the present invention.

- As used herein, the term "producer cell" or "vector producing cell" refers to a cell
5 which contains all the elements necessary for production of regulated retroviral vector particles and regulated retroviral delivery systems.

Preferably, the producer cell is obtainable from a stable producer cell line.

- 10 Preferably, the producer cell is obtainable from a derived stable producer cell line.

Preferably, the producer cell is obtainable from a derived producer cell line

- 15 As used herein, the term "derived producer cell line" is a transduced producer cell line which has been screened and selected for high expression of a marker gene. Such cell lines contain retroviral insertions in integration sites that support high level expression from the retroviral genome. The term "derived producer cell line" is used interchangeably with the term "derived stable producer cell line" and
20 the term "stable producer cell line"

Preferably the derived producer cell line includes but is not limited to a retroviral and/or a lentiviral producer cell.

- 25 Preferably the derived producer cell line is an HIV or EIAV producer cell line, more preferably an EIAV producer cell line.

Preferably the envelope protein sequences, and nucleocapsid sequences are all stably integrated in the producer and/or packaging cell. However, one or more of

these sequences could also exist in episomal form and gene expression could occur from the episome.

5 As used herein, the term "packaging cell" refers to a cell which contains those elements necessary for production of infectious recombinant virus which are lacking in a recombinant viral vector. Typically, such packaging cells contain one or more vectors which are capable of expressing viral structural proteins (such as codon optimised *gag-pol* and *env*) but they do not contain a packaging signal.

10

The term "packaging signal" which is referred to interchangeably as "packaging sequence" or "*psi*" is used in reference to the non-coding, *cis*-acting sequence required for encapsidation of retroviral RNA strands during viral particle formation. In HIV-1, this sequence has been mapped to loci extending from
15 upstream of the major splice donor site (SD) to at least the *gag* start codon.

Packaging cell lines suitable for use with the above-described vector constructs may be readily prepared (see also WO 92/05266), and utilised to create producer cell lines for the production of retroviral vector particles. As already mentioned, a
20 summary of the available packaging lines is presented in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 449).

Also as discussed above, simple packaging cell lines, comprising a provirus in
25 which the packaging signal has been deleted, have been found to lead to the rapid production of undesirable replication competent viruses through recombination. In order to improve safety, second generation cell lines have been produced wherein the 3'LTR of the provirus is deleted. In such cells, two recombinations would be necessary to produce a wild type virus. A further improvement involves
30 the introduction of the *gag-pol* genes and the *env* gene on separate constructs so-

called third generation packaging cell lines. These constructs are introduced sequentially to prevent recombination during transfection (26; 27).

Preferably, the packaging cell lines are second generation packaging cell lines.

5

Preferably, the packaging cell lines are third generation packaging cell lines.

In these split-construct, third generation cell lines, a further reduction in recombination may be achieved by "codon wobbling". This technique, based on the redundancy of the genetic code, aims to reduce homology between the separate constructs, for example between the regions of overlap in the *gag-pol* and *env* open reading frames.

The packaging cell lines are useful for providing the gene products necessary to encapsidate and provide a membrane protein for a high titre regulated retrovirus vector and regulated nucleic gene delivery vehicle production. When regulated retrovirus sequences are introduced into the packaging cell lines, such sequences are encapsidated with the nucleocapsid (*gag-pol*) proteins and these units then bud through the cell membrane to become surrounded in cell membrane and to contain the envelope protein produced in the packaging cell line. These infectious regulated retroviruses are useful as infectious units *per se* or as gene delivery vectors.

The packaging cell may be a cell cultured *in vitro* such as a tissue culture cell line. Suitable cell lines include but are not limited to mammalian cells such as murine fibroblast derived cell lines or human cell lines. Preferably the packaging cell line is a human cell line, such as for example: HEK293, 293-T, TE671, HT1080.

Alternatively, the packaging cell may be a cell derived from the individual to be treated such as a monocyte, macrophage, blood cell or fibroblast. The cell may be isolated from an individual and the packaging and vector components administered *ex vivo* followed by re-administration of the autologous packaging cells.

It is highly desirable to use high-titre virus preparations in both experimental and practical applications. Techniques for increasing viral titre include using a *psi* plus packaging signal as discussed above and concentration of viral stocks. In addition, the use of different envelope proteins, such as the G protein from vesicular-stomatitis virus has improved titres following concentration to 10^9 per ml (28). However, typically the envelope protein will be chosen such that the viral particle will preferentially infect cells that are infected with the virus which it desired to treat. For example where an HIV vector is being used to treat HIV infection, the *env* protein used will be the HIV *env* protein.

The process of producing a retroviral vector in which the envelope protein is not the native envelope of the retrovirus is known as "pseudotyping". Certain envelope proteins, such as MLV envelope protein and vesicular stomatitis virus G (VSV-G) protein, pseudotype retroviruses very well. Pseudotyping is not a new phenomenon and examples may be found in WO-A-98/05759, WO-A-98/05754, WO-A-97/17457, WO-A-96/09400, WO-A-91/00047 and (29).

As used herein, the term "high titre" means an effective amount of a retroviral vector or particle which is capable of transducing a target site such as a cell.

As used herein, the term "effective amount" means an amount of a regulated retroviral or lentiviral vector or vector particle which is sufficient to induce expression of an NOI at a target site.

Preferably the titre is from at least 10^6 retrovirus particles per ml, such as from 10^6 to 10^7 per ml, more preferably at least 10^7 retrovirus particles per ml.

In accordance with the present invention, it is possible to manipulate the viral
5 genome or the regulated retroviral vector nucleotide sequence, so that viral genes
are replaced or supplemented with one or more NOIs which may be heterologous
NOIs.

The term "heterologous" refers to a nucleic acid sequence or protein sequence
10 linked to a nucleic acid or protein sequence which it is not naturally linked.

With the present invention, the term NOI (i.e. nucleotide sequence of interest)
includes any suitable nucleotide sequence, which need not necessarily be a
complete naturally occurring DNA sequence. Thus, the DNA sequence can be,
15 for example, a synthetic DNA sequence, a recombinant DNA sequence (i.e.
prepared by use of recombinant DNA techniques), a cDNA sequence or a partial
genomic DNA sequence, including combinations thereof. The DNA sequence
need not be a coding region. If it is a coding region, it need not be an entire

Many different selectable markers have been used successfully in retroviral vectors. These are reviewed in "Retroviruses" (1997 Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 444) and include, but are not limited to, the bacterial neomycin (neo) and hygromycin phosphotransferase genes which confer resistance to G418 and hygromycin respectively; a mutant mouse dihydrofolate reductase gene which confers resistance to methotrexate; the bacterial *gpt* gene which allows cells to grow in medium containing mycophenolic acid, xanthine and aminopterin; the bacterial *hisD* gene which allows cells to grow in medium without histidine but containing histidinol; the multidrug resistance gene (*mdr*) which confers resistance to a variety of drugs; and the bacterial genes which confer resistance to puromycin or phleomycin. All of these markers are dominant selectable and allow chemical selection of most cells expressing these genes. Other selectable markers are not dominant in that their use must be in conjunction with a cell line that lacks the relevant enzyme activity. Examples of non-dominant selectable markers include the thymidine kinase (*tk*) gene which is used in conjunction with *tk* cell lines.

Particularly preferred markers are blasticidin and neomycin, optionally operably linked to a thymidine kinase coding sequence typically under the transcriptional control of a strong viral promoter such the SV40 promoter.

In accordance with the present invention, suitable NOI sequences include those that are of therapeutic and/or diagnostic application such as, but are not limited to: sequences encoding cytokines, chemokines, hormones, antibodies, engineered immunoglobulin-like molecules, a single chain antibody, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, anti-sense RNA, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a tumour suppressor protein and growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives thereof (such as with an associated reporter group).

When included, such coding sequences may be typically operatively linked to a suitable promoter, which may be a promoter driving expression of a ribozyme(s), or a different promoter or promoters, such as in one or more specific cell types.

- 5 Suitable NOIs for use in the invention in the treatment or prophylaxis of cancer include NOIs encoding proteins which: destroy the target cell (for example a ribosomal toxin), act as: tumour suppressors (such as wild-type p53); activators of anti-tumour immune mechanisms (such as cytokines, co-stimulatory molecules and immunoglobulins); inhibitors of angiogenesis; or which provide enhanced
10 drug sensitivity (such as pro-drug activation enzymes); indirectly stimulate destruction of target cell by natural effector cells (for example, strong antigen to stimulate the immune system or convert a precursor substance to a toxic substance which destroys the target cell (for example a prodrug activating enzyme).

15

- Examples of prodrugs include but are not limited to etoposide phosphate (used with alkaline phosphatase; 5-fluorocytosine (with cytosine deaminase); Doxorubin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase); Para-N-bis (2-chloroethyl)aminobenzoyl glutamate (with Carboxypeptidase G2);
20 Cephalosporin nitrogen mustard carbamates (with B-lactamase); SR4233 (with p450 reductase); Ganciclovir (with HSV thymidine kinase); mustard pro-drugs with nitroreductase and cyclophosphamide or ifosfamide (with cytochrome p450).

- 25 Suitable NOIs for use in the treatment or prevention of ischaemic heart disease include NOIs encoding plasminogen activators. Suitable NOIs for the treatment or prevention of rheumatoid arthritis or cerebral malaria include genes encoding anti-inflammatory proteins, antibodies directed against tumour necrosis factor (TNF) alpha, and anti-adhesion molecules (such as antibody molecules or
30 receptors specific for adhesion molecules).

- The expression products encoded by the NOIs may be proteins which are secreted from the cell. Alternatively the NOI expression products are not secreted and are active within the cell. In either event, it is preferred for the NOI
- 5 expression product to demonstrate a bystander effect or a distant bystander effect; that is the production of the expression product in one cell leading to the killing of additional, related cells, either neighbouring or distant (e.g. metastatic), which possess a common phenotype. Encoded proteins could also destroy bystander tumour cells (for example with secreted antitumour antibody-ribosomal toxin
- 10 fusion protein), indirectly stimulated destruction of bystander tumour cells (for example cytokines to stimulate the immune system or procoagulant proteins causing local vascular occlusion) or convert a precursor substance to a toxic substance which destroys bystander tumour cells (eg an enzyme which activates a prodrug to a diffusible drug). Also, the delivery of NOI(s) encoding antisense
- 15 transcripts or ribozymes which interfere with expression of cellular genes for tumour persistence (for example against aberrant *myc* transcripts in Burkitts lymphoma or against *bcr-abl* transcripts in chronic myeloid leukemia. The use of combinations of such NOIs is also envisaged.
- 20 The NOI or NOIs of the present invention may also comprise one or more cytokine-encoding NOIs. Suitable cytokines and growth factors include but are not limited to: ApoE, Apo-SAA, BDNF, Cardiotrophin-1, EGF, ENA-78, Eotaxin, Eotaxin-2, Exodus-2, FGF-acidic, FGF-basic, fibroblast growth factor-10 (30). FLT3 ligand, Fractalkine (CX3C), GDNF, G-CSF, GM-CSF, GF- β 1,
- 25 insulin, IFN- γ , IGF-I, IGF-II, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8 (72 a.a.), IL-8 (77 a.a.), IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18 (IGIF), Inhibin α , Inhibin β , IP-10, keratinocyte growth factor-2 (KGF-2), KGF, Leptin, LIF, Lymphotoxin, Mullerian inhibitory substance, monocyte colony inhibitory factor, monocyte attractant protein (30 *ibid*), M-CSF, MDC (67
- 30 a.a.), MDC (69 a.a.), MCP-1 (MCAF), MCP-2, MCP-3, MCP-4, MDC (67 a.a.),

MDC (69 a.a.), MIG, MIP-1 α , MIP-1 β , MIP-3 α , MIP-3 β , MIP-4, myeloid progenitor inhibitor factor-1 (MPIF-1), NAP-2, Neurturin, Nerve growth factor, β -NGF, NT-3, NT-4, Oncostatin M, PDGF-AA, PDGF-AB, PDGF-BB, PF-4, RANTES, SDF1 α , SDF1 β , SCF, SCGF, stem cell factor (SCF), TARC, TGF- α ,
5 TGF- β , TGF- β 2, TGF- β 3, tumour necrosis factor (TNF), TNF- α , TNF- β , TNIL-1, TPO, VEGF, GCP-2, GRO/MGSA, GRO- β , GRO- γ , HCC1, 1-309.

The NOI or NOIs may be under the expression control of an expression regulatory element, such as a promoter and/or a promoter enhancer as known as
10 "responsive elements" in the present invention.

When the regulated retroviral vector particles are used to transfer NOIs into cells which they transduce, such vector particles also designated "viral delivery systems" or "retroviral delivery systems". Viral vectors, including retroviral
15 vectors, have been used to transfer NOIs efficiently by exploiting the viral transduction process. NOIs cloned into the retroviral genome can be delivered efficiently to cells susceptible to transduction by a retrovirus. Through other genetic manipulations, the replicative capacity of the retroviral genome can be destroyed. The vectors introduce new genetic material into a cell but are unable
20 to replicate.

The regulated retroviral vector of the present invention can be delivered by viral or non-viral techniques. Non-viral delivery systems include but are not limited to DNA transfection methods. Here, transfection includes a process using a non-
25 viral vector to deliver a gene to a target mammalian cell.

Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial
30 amphiphiles (CFAs) (31), multivalent cations such as spermine, cationic lipids or

polylysine, 1, 2,-bis (oleoyloxy)-3-(trimethylammonio) propane (DOTAP)-cholesterol complexes (32) and combinations thereof.

5 Viral delivery systems include but are not limited to adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, a retroviral vector, a lentiviral vector, or a baculoviral vector. These viral delivery systems may be configured as a split-intron vector. A split intron vector is described in WO 99/15683.

10 Other examples of vectors include *ex vivo* delivery systems, which include but are not limited to DNA transfection methods such as electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection.

15 The vector may be a plasmid DNA vector. Alternatively, the vector may be a recombinant viral vector. Suitable recombinant viral vectors include adenovirus vectors, adeno-associated viral (AAV) vectors, Herpes-virus vectors, or retroviral vectors, lentiviral vectors or a combination of adenoviral and lentiviral vectors. In the case of viral vectors, gene delivery is mediated by viral infection of a target cell.

20 If the features of adenoviruses are combined with the genetic stability of retro/lentiviruses then essentially the adenovirus can be used to transduce target cells to become transient retroviral producer cells that could stably infect neighbouring cells.

25 The present invention also provides a pharmaceutical composition for treating an individual by gene therapy, wherein the composition comprises a therapeutically effective amount of a regulated retroviral vector according to the present invention. The pharmaceutical composition may be for human or animal usage.

30 Typically, a physician will determine the actual dosage which will be most

suitable for an individual subject and it will vary with the age, weight and response of the particular patient.

The composition may optionally comprise a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: minipumps, inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The present invention is believed to have a wide therapeutic applicability - depending on *inter alia* the selection of the one or more NOIs.

For example, the present invention may be useful in the treatment of the disorders listed in WO-A-98/05635. For ease of reference, part of that list is now provided: cancer, inflammation or inflammatory disease, dermatological disorders, fever, cardiovascular effects, haemorrhage, coagulation and acute phase response, cachexia, anorexia, acute infection, HIV infection, shock states, graft-versus-host reactions, autoimmune disease, reperfusion injury, meningitis, migraine and aspirin-dependent anti-thrombosis; tumour growth, invasion and spread, angiogenesis, metastases, malignant, ascites and malignant pleural effusion; cerebral ischaemia, ischaemic heart disease, osteoarthritis, rheumatoid arthritis, osteoporosis, asthma, multiple sclerosis, neurodegeneration, Alzheimer's disease, atherosclerosis, stroke, vasculitis, Crohn's disease and ulcerative colitis; periodontitis, gingivitis; psoriasis, atopic dermatitis, chronic ulcers, epidermolysis bullosa; corneal ulceration, retinopathy and surgical wound healing; rhinitis, allergic conjunctivitis, eczema, anaphylaxis; restenosis, congestive heart failure, endometriosis, atherosclerosis or endosclerosis.

In addition, or in the alternative, the present invention may be useful in the treatment of disorders listed in WO-A-98/07859. For ease of reference, part of that list is now provided: cytokine and cell proliferation/differentiation activity; immunosuppressant or immunostimulant activity (e.g. for treating immune deficiency, including infection with human immune deficiency virus; regulation of lymphocyte growth; treating cancer and many autoimmune diseases, and to prevent transplant rejection or induce tumour immunity); regulation of haematopoiesis, e.g. treatment of myeloid or lymphoid diseases; promoting growth of bone, cartilage, tendon, ligament and nerve tissue, e.g. for healing wounds, treatment of burns, ulcers and periodontal disease and neurodegeneration; inhibition or activation of follicle-stimulating hormone (modulation of fertility); chemotactic/chemokinetic activity (e.g. for mobilising specific cell types to sites of injury or infection); haemostatic and thrombolytic activity (e.g. for treating haemophilia and stroke); antiinflammatory activity (for

treating e.g. septic shock or Crohn's disease); as antimicrobials; modulators of e.g. metabolism or behaviour; as analgesics; treating specific deficiency disorders; in treatment of e.g. psoriasis, in human or veterinary medicine.

- 5 In addition, or in the alternative, the present invention may be useful in the treatment of disorders listed in WO-A-98/09985. For ease of reference, part of that list is now provided: macrophage inhibitory and/or T cell inhibitory activity and thus, anti-inflammatory activity; anti-immune activity, i.e. inhibitory effects against a cellular and/or humoral immune response, including a response not
- 10 associated with inflammation; inhibit the ability of macrophages and T cells to adhere to extracellular matrix components and fibronectin, as well as up-regulated fas receptor expression in T cells; inhibit unwanted immune reaction and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus
- 15 erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of
- 20 the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididymo-orchitis, infertility, orchidal trauma or other immune-related testicular
- 25 diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia,
- 30 scleritis, retinitis pigmentosa, immune and inflammatory components of

degenerative fondus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and
5 other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia
10 complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy,
15 subacute neuropathy, chronic neuropathy, Guillain-Barre syndrome, Sydenham chora, myasthenia gravis, pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and
20 inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery, bone marrow transplantation or other transplantation complications and/or side effects, inflammatory and/or immune complications and side effects of gene therapy, e.g.
25 due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells,
30 tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers,

natural or artificial skin tissue.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention. The Examples refer to the Figures. In the Figures:

Description of the Figures

10 Figure 1 shows schematically how to create a suitable 3' LTR by PCR;

Figure 2 shows the codon usage table for wild type HIV *gag-pol* of strain HXB2 (accession number: K03455);

15 Figure 3a shows the codon usage table of the codon optimised sequence designated gagpol-SYNGp. Figure 3b shows a comparative codon usage table;

Figure 4 shows the codon usage table of the wild type HIV *env* called env-mn;

20 Figure 5 shows the codon usage table of the codon optimised sequence of HIV *env* designated SYNGp160mn;

Figure 6 shows two plasmid constructs for use in the invention;

25 Figure 7 shows the principle behind two systems for producing retroviral vector particles;

Figure 8 shows a sequence comparison between the wild type HIV *gag-pol* sequence (pGP-RRE3) and the codon optimised *gag-pol* sequence (pSYNGP);

30

Figure 9 shows a sequence comparison between the wild type ELAV *gag-pol* sequence (WT) and the codon optimised *gag-pol* sequence (CO);

Figure 10 shows Rev independence of protein expression particle formation;

5

Figure 11 shows translation rates of wild-type (WT) and codon optimised *gag-pol*;

Figure 12 shows *gag-pol* mRNA levels in total and cytoplasmic fractions;

10 Figure 13 shows the effect of insertion of WT *gag* downstream of the codon optimised gene on RNA and protein levels;

Figure 14 shows the plasmids used to study the effect of HIV-1 *gag* on the codon optimised gene;

15

Figure 15 shows the effect on cytoplasmic RNA of insertion of HIV-1 *gag* upstream of the codon optimised gene;

Figure 16 shows the effect of Leptomycin B (LMB) on protein production;

20

Figure 17 shows the cytoplasmic RNA levels of the vector genomes;

Figure 18 shows transduction efficiency at MOI 1;

25 Figure 19 shows a schematic representation of pGP-RRE3;

Figure 20 shows a schematic representation of pSYNGP;

Figure 21 shows vector titres generated with different *gag-pol* constructs;

30

Figure 22 shows vector titres from the Rev/RRE (-) and (+) genomes;

Figure 23 shows vector titres from the pHS series of vector genomes;

- 5 Figure 24 shows vector titres for the pHS series of vector genomes in the presence or absence of Rev/RRE;

Figure 25 shows an analysis of *gag-pol* constructs;

- 10 Figure 26 shows a Western blot of 293T extracts;

Figure 27 is a schematic representation of pESYNGP;

Figure 28 is a schematic representation of LpESYNGP;

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Figure 29 is a schematic representation of LpESYNGPRRE;

Figure 30 is a schematic representation of pESYNGPRRE;

- 20 Figure 31 is a schematic representation of pONY4.0Z;

Figure 32 is a schematic representation of pONY8.0Z;

Figure 33 is a schematic representation of pONY8.1Z;

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Figure 34 is a schematic representation of pONY3.1;

Figure 35 is a schematic representation of pCIneoERev;

- 30 Figure 36 is a schematic representation of pESYNREV;

Figures 37 and 38 show the effect of different vector constructs on viral vector titres;

5 Figures 39 and 40 show the effect of different vector constructs on RT activity;

Figure 41 shows the effect of the 5' leader sequence on viral vector titre;

Figure 42 shows viral vector titres when using pONY8.1Z;

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Figure 43 shows a comparison between the sequences of pONY3.1 and codon optimised pONY3.2OPTI in the first 372 nucleotides of *gag*;

Figure 44 is a schematic representation of pIRES1hygESYNGP;

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Figure 45 and 46 show the results of experiments to confirm that codon optimised *gag-pol* can be used in the production of packaging and producer cell lines;

20 Figures 47 and 48 show the results of experiments which confirm that RNA from codon optimised *gag-pol* is packaged less efficiently than that from the wild-type gene;

Figure 49 shows the results of an experiment which confirms that expression from pESYNGP and pESDSYNGP are similar;

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Figure 50 is a schematic representation of pESDSYNGP; and

Figure 51 shows the results of an experiment which confirms that the efficiency of encapsidating *gag-pol* RNA in PEV-17 cells and B-241 cells is similar.

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In more detail, Figure 8 shows a sequence comparison between the wild type HIV *gag-pol* sequence (pGP-RRE3) and the codon optimised *gag-pol* sequence (pSYNGP) wherein the upper sequence represents pSYNGP and the lower sequence represents pGP-RRE3.

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Figure 10 shows Rev independence of protein expression particle formation. 5µg of the *gag-pol* expression plasmids were transfected into 293T cells in the presence or absence of Rev (pCMV-Rev, 1µg) and protein levels were determined 48 hours post transfection in culture supernatants (A) and cell lysates (B). HIV-1 positive human serum was used to detect the *gag-pol* proteins. The blots were re-probed with an anti-actin antibody, as an internal control (C). The protein marker (New England Biolabs) sizes (in kDa) are shown on the side of the gel. Lanes: 1. Mock transfected 293T cells, 2. pGP-RRE3, 3. pGP-RRE3 + pCMV-Rev, 4. pSYNGP, 5. pSYNGP + pCMV-Rev, 6. pSYNGP-RRE, 7. pSYNGP-RRE + pCMV-Rev, 8. pSYNGP-ERR, 9. pSYNGP-ERR + pCMV-Rev.

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Figure 11 shows translation rates of WT and codon optimised *gag-pol*. 293T cells were transfected with 2µg pGP-RRE3 (+/- 1µg pCMV-Rev) or 2µg pSYNGP. Protein samples from culture supernatants (A) and cell extracts (B) were analysed by Western blotting 12, 25, 37 and 48 hours post-transfection. HIV-1 positive human serum was used to detect *gag-pol* proteins (A, B) and an anti-actin antibody was used as an internal control (C). The protein marker sizes are shown on the side of the gel (in kD). A Phosphorimager was used for quantification of the results. Lanes: 1. pGP-RRE3 12h, 2. pGP-RRE3 25h, 3. pGP-RRE3 37h, 4. pGP-RRE3 48h, 5. pGP-RRE3 + pCMV-Rev 12h, 6. pGP-RRE3 + pCMV-Rev 25h, 7. pGP-RRE3 + pCMV-Rev 37h, 8. pGP-RRE3 + pCMV-Rev 48h, 9. pSYNGP 12h, 10. pSYNGP 25h, 11. pSYNGP 37h, 12. pSYNGP 48h, 13. Mock transfected 293T cells.

25

Figure 12 shows *gag-pol* mRNA levels in total and cytoplasmic fractions. Total and cytoplasmic RNA was extracted from 293T cells 36 hours after transfection with 5µg of the *gag-pol* expression plasmid (+/- 1µg pCMV-Rev) and mRNA levels were estimated by Northern blot analysis. A probe complementary to nt 1222-1503 of both the wild type and codon optimised gene was used. Panel A shows the band corresponding to the HIV-1 *gag-pol*. The sizes of the mRNAs are 4.4 kb for the codon optimised and 6 kb for the wild type gene. Panel B shows the band corresponding to human ubiquitin (internal control for normalisation of results). Quantification was performed using a Phosphorimager. Lane numbering: c indicates cytoplasmic fraction and t indicates total RNA fraction. Lanes: 1. pGP-RRE3 , 2. pGP-RRE3 + pCMV-Rev, 3. pSYNGP, 4. pSYNGP + pCMV-Rev, 5. pSYNGP-RRE, 6. pSYNGP-RRE + pCMV-Rev, 7. Mock transfected 293T cells, 8. pGP-RRE3 + pCMV-Rev, 9. Mock transfected 293T cells, 10. pSYNGP.

Figure 13 shows the effect of insertion of WT *gag* downstream of the codon optimised gene on RNA and protein levels. The wt *gag* sequence was inserted downstream of the codon optimised gene in both orientations (*NotI* site), resulting in plasmids pSYN6 (correct orientation, see Figure 14) and pSYN7 (reverse orientation, see Figure 14). The gene encoding for β-galactosidase (LacZ) was also inserted in the same site and the correct orientation (plasmid pSYN8, see Figure 14). 293T cells were transfected with 5 µg of each plasmid and 48 hours post transfection mRNA and protein levels were determined as previously described by means of Northern and Western blot analysis respectively.

Northern blot analysis in cytoplasmic RNA fractions. The blot was probed with a probe complementary to nt 1510-2290 of the codon optimised gene (I) and was re-probed with a probe specific for human ubiquitin (II). Lanes: 1. pSYNGP, 2. pSYN8, 3. pSYN7, 4. pSYN6

Western blot analysis: HIV-1 positive human serum was used to detect the gag-pol proteins (I) and an anti-actin antibody was used as an internal control (II). Lanes: Cell lysates: 1. Mock transfected 293T cells, 2. pGP-RRE3 + pCMV-Rev, 3. pSYNGP, 4. pSYN6, 5. pSYN7, 6. pSYN8. Supernatants: 7. Mock transfected 293T cells, 8. pGP-RRE3 + pCMV-Rev, 9. pSYNGP, 10. pSYN6, 11. pSYN7, 12. pSYN8. The protein marker (New England Biolabs) sizes are shown on the side of the gel.

Figure 14 shows the plasmids used to study the effect of HIV-1 *gag* on the codon optimised gene. The backbone for all constructs was pCI-Neo. Syn *gp*: The codon optimised HIV-1 *gag-pol* gene. HXB2 *gag*: The wild type HIV-1 *gag* gene. HXB2 *gag,r*: The wild type HIV-1 *gag* gene in the reverse orientation. HXB2 *gag*ΔATG: The wild type HIV-1 *gag* gene without the *gag* ATG. HXB2 *gag-fr.sh.*: The wild type HIV-1 *gag* gene with a frameshift mutation. HXB2 *gag* 625-1503: Nucleotides 625-1503 of the wild type HIV-1 *gag* gene. HXB2 *gag* 1-625: Nucleotides 1-625 of the wild type HIV-1 *gag* gene.

Figure 15 shows the effect on cytoplasmic RNA of insertion of HIV-1 *gag* upstream of the codon optimised gene. Cytoplasmic RNA was extracted 48 hours post transfection of 293T cells (5 µg of each pSYN plasmid was used and 1 µg of pCMV-Rev was co-transfected in some cases). The probe that was used was designed to be complementary to nt 1510-2290 of the codon optimised gene (I). A probe specific for human ubiquitin was used as an internal control (II). Lanes: 1. pSYNGP, 2. pSYN9, 3. pSYN10, 4. pSYN10 + pCMV-Rev, 5. pSYN11, 6. pSYN11 + pCMV-Rev, 7. pCMV-Rev. Lanes: 1. pSYNGP, 2. pSYNGP-RRE, 3. pSYNGP-RRE + pCMV-Rev, 4. pSYN12, 5. pSYN14, 6. pSYN14 + pCMV-Rev, 7. pSYN13, 8. pSYN15, 9. pSYN17, 10. pGP-RRE3, 11. pSYN6, 12. pSYN9, 13. pCMV-Rev.

Figure 16 shows the effect of LMB on protein production. 293T cells were transfected with 1µg pCMV-Rev and 3µg of pGP-RRE3/pSYNGP/pSYNGP-RRE (+/- 1µg pCMV-Rev). Transfections were done in duplicate. 5 hours post transfection the medium was replaced with fresh medium in the first set and with
5 fresh medium containing 7.5 nM LMB in the second. 20 hours later the cells were lysed and protein production was estimated by Western blot analysis. HIV-1 positive human serum was used to detect the gag-pol proteins (A) and an anti-actin antibody was used as an internal control (B). Lanes: 1. pGP-RRE3, 2. pGP-RRE3 + LMB, 3. pGP-RRE3 + pCMV-Rev, 4. pGP-RRE3 + pCMV-Rev +
10 LMB, 5. pSYNGP, 6. pSYNGP + LMB, 7. pSYNGP + pCMV-Rev, 8. pSYNGP + pCMV-Rev + LMB, 9. pSYNGP-RRE, 10. pSYNGP-RRE + LMB, 11. pSYNGP-RRE + pCMV-Rev, 12. pSYNGP-RRE + pCMV-Rev + LMB.

Figure 17 shows the cytoplasmic RNA levels of the vector genomes. 293T cells
15 were transfected with 10 µg of each vector genome. Cytoplasmic RNA was extracted 48 hours post transfection. 20 µg of RNA were used from each sample for Northern blot analysis. The 700bp probe was designed to hybridise to all vector genome RNAs (see Materials and Methods). Lanes: 1. pH6nZ, 2. pH6nZ + pCMV-Rev, 3. pH6.1nZ, 4. pH6.1nZ + pCMV-Rev, 5. pHS1nZ, 6. pHS2nZ, 7.
20 pHS3nZ, 8. pHS4nZ, 9. pHS5nZ, 10. pHS6nZ, 11. pHS7nZ, 12. pHS8nZ, 13. pCMV-Rev.

Figure 18 shows transduction efficiency at MOI 1. Viral stocks were generated by co-transfection of each *gag-pol* expression plasmid (5 or 0.5 µg), 15 µg
25 pH6nZ or pHS3nZ (vector genome plasmid) and 5 µg pHCMVG (VSV envelope expression plasmid) on 293T cells. Virus was concentrated as previously described (45) and transduction efficiency was determined at m.o.i.'s 0.01-1 on HT1080 cells. There was a linear correlation of transduction efficiency and m.o.i. in all cases. An indicative picture at m.o.i. 1 is shown here. Transduction
30 efficiency was >80% with either genome, either *gag-pol* and either high or low

amounts of pSYNGP. Titres before concentration (I.U./ml): on 293T cells: A. 6.6×10^5 , B. 7.6×10^5 , C. 9.2×10^5 , D. 1.5×10^5 , on HT1080 cells: A. 6.0×10^4 , B. 9.9×10^4 , C. 8.0×10^4 , D. 2.9×10^4 . Titres after concentration (I.U./ml) on HT1080 cells: A. 6.0×10^5 , B. 2.0×10^6 , C. 1.4×10^6 , D. 2.0×10^5 .

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Figure 21 shows vector titers obtained with differed *gag-pol* constructs. Viral stocks were generated by co-transfection of each *gag-pol* expression plasmid, pH6nZ (vector genome plasmid) and pHCMVG (VSV envelope expression plasmid, 2.5 μ g for each transfection) on 293T cells. Titres (I.U./ml of virus stock) were measured on 293T cells by counting the number of blue colonies following X-Gal staining 48 hours after transduction. Experiments were performed at least twice and the variation between experiments was less than 15%.

Figure 22 shows vector titres from the Rev/RRE (-) and (+) genomes. The retroviral vectors were generated as described in the Examples. Titres (I.U./ml of viral stock + SD) were determined in 293T cells.

Figure 23 shows vector titres from the pHS series of vector genomes. The retroviral vector was generated as described in the Examples. Titres (I.U./ml of viral stock + SD) were determined in 293T cells. Rev is provided from pCMV-Rev. Note that pH6nZ expresses Rev and contains the RRE. None of the other genomes express Rev or contain the RRE. Expression from pSYNGP is Rev independent, whereas it is Rev dependent for pGP-RRE3.

Figure 24 shows vector titres for the pHS series of vector genomes in the presence or absence of Rev/RRE. The retroviral vector was generated as described in the Examples. 5 μ g of vector genome, 5 μ g of pSYNGP and 2.5 μ g of pHCMVG were used and titres (I.U./ml) were determined in 293T cells. Experiments were performed at least twice and the variation between experiments was less than 15%. Rev is provided from pCMV-Rev (1 μ g). Note that pH6nZ expresses Rev and

contains the RRE. None of the pHS genomes expresses Rev and only pHS1nZR, pHS3nZR, pHS7nZR and pH6.1nZR contain the RRE. gag-pol expression from pSYNGP is Rev independent.

- 5 Figure 26 shows a Western blot of 293T extracts wherein 30:g of total cellular protein was separated by SDS/Page electrophoresis, transferred to nitro-cellulose and probed with anti ELAV antibodies. The secondary antibody was anti-Horse HRP (Sigma).

- 10 In Figure 38 the titres are shown in lacZ forming units (L.F.U.)/ml. The vectors used are indicated in boxes above the bars.
 For ease of reference, we also set out the sequences listed in the accompanying Sequence Listing:
 SEQ ID NO:1 shows the sequence of the wild-type *gag-pol* sequence for the strain
 15 HXB2 (accession no. K03455);
 SEQ ID NO:2 shows the sequence of pSYNGP;
 SEQ ID NO:3 shows the sequence of the Envelope gene for HIV-1 MN (Genbank accession no. M17449);
 SEQ ID NO:4 shows the sequence of SYNgp-160mn – codon optimised env
 20 sequence;
 SEQ ID NO:5 shows the sequence of pESYNGP;
 SEQ ID NO:6 shows the sequence of LpESYNGP;
 SEQ ID NO:7 shows the sequence of pESYNGPRRE;
 SEQ ID NO:8 shows the sequence of LpESYNGPRRE;
 25 SEQ ID NO:9 shows the sequence of pONY4.0Z;
 SEQ ID NO:10 shows the sequence of pONY8.0Z;
 SEQ ID NO:11 shows the sequence of pONY8.1Z;
 SEQ ID NO:12 shows the sequence of pONY3.1;
 SEQ ID NO:13 shows the sequence of pCIneoERev;
 30 SEQ ID NO:14 shows the sequence of pESYNREV;

- SEQ ID NO:15 shows the sequence of codon optimised HIV *gag-pol*;
SEQ ID NO:16 shows the sequence of codon optimised EIAV *gag-pol*;
SEQ ID NO:17 shows the sequence of pIRES1hygESYNGP;
SEQ ID NO:18 shows the sequence of pESDSYNGP; and
5 SEQ ID NO:19 shows the sequence of pONY8.3G FB29(-).

Example 1 - HIV

Cell lines

- 10 293T cells (33) and HeLa cells (34) were maintained in Dubecco's modified Eagle's medium containing 10% (v/v) fetal calf serum and supplemented with L-glutamine and antibiotics (penicillin-streptomycin). 293T cells were obtained from D. Baltimore (Rockefeller University).

15 HIV- 1 proviral clones

Proviral clones pWI3 (35) and pNL4-3 (36) were used.

Construction of a Packaging System

20

- In one of the present examples, a modified codon optimised HIV *env* sequence is used (SEQ I.D. No. 4). The corresponding *env* expression plasmid is designated pSYNgp160mn. The modified sequence contains extra motifs not used by (37). The extra sequences were taken from the HIV *env* sequence of strain MN and codon
25 optimised. Any similar modification of the nucleic acid sequence would function similarly as long as it used codons corresponding to abundant tRNAs (38).

Codon optimised HIV-1 *gag-pol* gene

A codon optimised *gag-pol* gene, shown from nt 1108 to 5414 of SEQ ID NO: 2 was constructed by annealing a series of short overlapping oligonucleotides (approximately 30-40mers with 25% overlap, i.e. approximately 9 nucleotides). Oligonucleotides were purchased from R&D SYSTEMS (R&D Systems Europe Ltd, 4-10 The Quadrant, Barton Lane, Abingdon, OX14 3YS, UK). Codon optimisation was performed using the sequence of HXB-2 strain (AC: K03455) (39). The Kozak consensus sequence for optimal translation initiation (40) was also included. A fragment from base 1222 from the beginning of *gag* until the end of *gag* (1503) was not optimised in order to maintain the frameshift site and the overlap between the *gag* and *pol* reading frames. This was from clone pNL4-3. (When referring to base numbers within the *gag-pol* gene base 1 is the A of the *gag* ATG, which corresponds to base 790 from the beginning of the HXB2 sequence. When referring to sequences outside the *gag-pol* then the numbers refer to bases from the beginning of the HXB2 sequence, where base 1 corresponds to the beginning of the 5' LTR). Some deviations from optimisation were made in order to introduce convenient restriction sites. The final codon usage is shown in Figure 3b, which now resembles that of highly expressed human genes and is quite different from that of the wild type HIV-1 *gag-pol*. The gene was cloned into the mammalian expression vector pCIneo (Promega) in the *EcoRI-NotI* sites. The resulting plasmid was named pSYNGP (Figure 20, SEQ ID No 2). Sequencing of the gene in both strands verified the absence of any mistakes. A sequence comparison between the codon optimised and wild type HIV *gag-pol* sequence is shown in Figure 8.

25

Rev/RRE constructs

The HIV-1 RRE sequence (bases 7769-8021 of the HXB2 sequence) was amplified by PCR from pWI3 proviral clone with primers bearing the *NotI* restriction site and was subsequently cloned into the *NotI* site of pSYNGP. The resulting plasmids

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were named pSYNGP-RRE (RRE in the correct orientation) and pSYNGP-ERR (RRE in the reverse orientation).

Pseudotyped viral particles

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In one form of the packaging system a synthetic *gag-pol* cassette is coexpressed with a heterologous envelope coding sequence. This could be for example VSV-G (44, 45), amphotropic MLV env (46, 47) or any other protein that would be incorporated into the HIV or EIAV particle (48). This includes molecules capable of targeting the vector to specific tissues.

10

HIV-1 Vector genome constructs

pH6nZ is derived from pH4Z (49) by the addition of a single nucleotide to place an extra guanine residue that was missing from pH4Z at the 5' end of the vector genome transcript to optimise reverse transcription. In addition the gene coding for β -galactosidase (LacZ) was replaced by a gene encoding for a nuclear localising β -galactosidase. (We are grateful to Enca Martin-Rendon and Said Ismail for providing pH6nZ). In order to construct Rev(-) genome constructs the following modifications were made : a) A 1.8 kb *Pst*I - *Pst*I fragment was removed from pH6nZ, resulting in plasmid pH6.1nZ and b) an *Eco*NI (filled) - *Sph*I fragment was substituted with a *Spe*I (filled) - *Sph*I fragment from the same plasmid (pH6nZ), resulting in plasmid pH6.2nZ. In both cases sequences within *gag* (nt 1-625) were retained, as they have been shown to play a role in packaging (93). *Rev*, RRE and any other residual *env* sequences were removed. pH6.2nZ further contains the *env* splice acceptor, whereas pH6.1nZ does not.

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A series of vectors encompassing further *gag* deletions plus or minus a mutant major splice donor (SD) (GT to CA mutation) were also derived from pH6Z. These were made by PCR with primers bearing a *Nar*I (5' primers) and an *Spe*I

(3' primers) site. The PCR products were inserted into pH6Z at the *NarI* - *SpeI* sites. The resulting vectors were named pHS1nZ (containing HIV-1 sequences up to *gag* 40), pHS2nZ (containing HIV-1 sequences up to *gag* 260), pHS3nZ (containing HIV-1 sequences up to *gag* 360), pHS4nZ (containing HIV-1 sequences up to *gag* 625), pHS5nZ (same as pHS1nZ but with a mutant SD), pHS6nZ (same as pHS2nZ but with a mutant SD), pHS7nZ (same as pHS3nZ but with a mutant SD) and pHS8nZ (same as pHS4nZ but with a mutant SD).

In addition, the RRE sequence (nt 7769-8021 of the HXB2 sequence) was inserted in the *SpeI* (filled) site of pH6.1nZ, pHS1nZ, pHS3nZ and pHS7nZ resulting in plasmids pH6.1nZR, pHS1nZR, pHS3nZR and pHS7nZR respectively.

Other modifications to the genome have been made including the generation of a SIN vector (by deletion of part of the 3' U3), the replacement of the LTRs with those from MLV or replacement of part of the 3'U3 with the MLV U3 region.

Transient transfections, transductions and determination of viral titres

These were performed as previously described (49, 50). Briefly, 293T cells were seeded on 6cm dishes and 24 hours later they were transiently transfected by overnight calcium phosphate treatment. The medium was replaced 12 hours post-transfection and unless otherwise stated supernatants were harvested 48 hours post-transfection, filtered (through 0.22 or 0.45 μ m filters) and titered by transduction of 293T cells. For this reason supernatant at appropriate dilutions of the original stock was added to 293T cells (plated onto 6 or 12 well plates 24 hours prior to transduction). 8 μ g/ml Polybrene (Sigma) was added to each well and 48 hours post transduction viral titres were determined by X-gal staining.

Luminescent β -galactosidase (β -gal) assays

These were performed on total cell extracts using a luminescent β -gal reporter system (CLONTECH). Untransfected 293T cells were used as negative control and
5 293T cells transfected with pCMV- β -gal (CLONTECH) were used as positive control.

RNA analysis

10 Total or cytoplasmic RNA was extracted from 293T cells by using the RNeasy mini kit (QUIAGEN) 36-48 hours post-transfection. 5-10 μ g of RNA was subjected to Northern blot analysis as previously described (51). Correct fractionation was verified by staining of the agarose gel. A probe complementary to bases 1222-1503 of the *gag-pol* gene was amplified by PCR from HIV-1 pNL4-3 proviral clone and
15 was used to detect both the codon optimised and wild type *gag-pol* mRNAs. A second probe, complementary to nt 1510-2290 of the codon optimised gene was also amplified by PCR from plasmid pSYNGP and was used to detect the codon optimised genes only. A 732 bp fragment complementary to all vector genomes used in this study was prepared by an *SpeI*-*AvrII* digestion of pH6nZ. A probe
20 specific for ubiquitin (CLONTECH) was used to normalise the results. All probes were labelled by random labelling (STRATAGENE) with α - 32 P dCTP (Amersham). The results were quantitated by using a Storm PhosphorImager (Molecular Dynamics) and shown in Figure 12. In the total cellular fractions the 47S rRNA precursor could be clearly seen, whereas it was absent from the cytoplasmic
25 fractions. As expected (52), Rev stimulates the cytoplasmic accumulation of wild type *gag-pol* mRNA (lanes 1c and 2c). RNA levels were 10-20 fold higher for the codon optimised gene compared to the wild type one, both in total and cytoplasmic fractions (compare lanes 3t-2t, 3c-2c, 10c-8c). The RRE sequence did not significantly destabilise the codon optimised RNAs since RNA levels were similar
30 for codon optimised RNAs whether or not they contained the RRE sequence

(compare lanes 3 and 5). Rev did not markedly enhance cytoplasmic accumulation of the codon optimised *gag-pol* mRNAs, even when they contained the RRE sequence (differences in RNA levels were less than 2-fold, compare lanes 3-4 or 5-6).

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It appeared from a comparison of Figures 10 and 12 that all of the increase in protein expression from *syngp* could be accounted for by the increase in RNA levels. In order to investigate whether this was due to saturating levels of RNA in the cell, we transfected 0.1, 1 and 10 μ g of the wild type or codon optimised expression vectors into 293T cells and compared protein production. In all cases protein production was 10-fold higher for the codon optimised gene for the same amount of transfected DNA, while increase in protein levels was proportional to the amount of transfected DNA for each individual gene. It seems likely therefore that the enhanced expression of the codon optimised gene can be mainly attributed to the enhanced RNA levels present in the cytoplasm and not to increased translation.

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Protein analysis

Total cell lysates were prepared from 293T cells 48 hours post-transfection (unless otherwise stated) with an alkaline lysis buffer. For extraction of proteins from cell supernatants the supernatant was first passed through a 0.22µm filter and the vector particles were collected by centrifugation of 1 ml of supernatant at 21,000 g for 30 minutes. Pellets were washed with PBS and then re-suspended in a small volume (2-10 µl) of lysis buffer. Equal protein amounts were separated on a SDS 10-12% (v/v) polyacrylamide gel. Proteins were transferred to nitrocellulose membranes which were probed sequentially with a 1:500 dilution of HIV-1 positive human serum (AIDS Reagent Project, ADP508, Panel E) and a 1:1000 dilution of horseradish peroxidase labelled anti-human IgG (Sigma, A0176). Proteins were visualised using the ECL or ECL-plus western blotting detection reagent (Amersham). To verify equal protein loading, membranes were stripped and re-probed with a 1:1000 dilution of anti-actin antibody (Sigma, A2066), followed by a 1:2000 dilution of horseradish peroxidase labelled anti-rabbit IgG (Vector Laboratories, PI-1000).

Expression of *gag-pol* gene products and vector particle production

The wild type *gag-pol* (pGP-RRE3 - Figure 19) (49), and codon optimised expression vectors (pSYNGP, pSYNGP-RRE and pSYNGP-ERR) were transiently transfected into 293T cells. Transfections were performed in the presence or absence of a Rev expression vector, pCMV-Rev (53), in order to assess Rev-dependence for expression. Western blot analysis was performed on cell lysates and supernatants to assess protein production. The results are shown in Figure 10. As expected (54), expression of the wild type gene is observed only when Rev is provided *in trans* (lanes 2 and 3). In contrast, when the codon optimised *gag-pol* was used, there was high level expression in both the presence and absence of Rev (lanes 4 and 5), indicating that in this system there was no

requirement for Rev. Protein levels were higher for the codon optimised gene than for the wild type *gag-pol* (compare lanes 4-9 with lane 3). The difference was more evident in the cell supernatants (approximately 10-fold higher protein levels for the codon optimised gene compared to the wild type one, quantitated by using a PhosphorImager) than in the cell lysates.

In previous studies where the RRE has been included in *gag-pol* expression vectors that had been engineered to remove INS sequences, inclusion of the RRE lead to a decrease in protein levels, that was restored by providing Rev *in trans* (55). In our hands, the presence of the RRE in the fully codon optimised *gag-pol* mRNA did not affect protein levels and provision of Rev *in trans* did not further enhance expression (lanes 6 and 7).

In order to compare translation rates between the wild type and codon optimised gene, protein production from the wild type and codon optimised expression vector was determined at several time intervals post transfection into 293T cells. Protein production and particle formation was determined by Western blot analysis and the results are shown in Figure 11. Protein production and particle formation was 10-fold higher for the codon optimised *gag-pol* at all time points.

To further determine whether this enhanced expression that was observed with the codon optimised gene was due to better translation or due to effects on the RNA, RNA analysis was carried out.

25 The efficiency of vector production using the codon optimised *gag-pol* gene

To determine the effects of the codon optimised *gag-pol* on vector production, we used an HIV vector genome, pH6nZ and the VSV-G envelope expression plasmid pHCMVG (113), in combination with either pSYNGP, pSYNGP-RRE, pSYNGP-ERR or pGP-RRE3 as a source for the *gag-pol* in a plasmid ratio of 2 :

1 : 2 in a 3 plasmid co-transfection of 293T cells (49). Whole cell extracts and culture supernatants were evaluated by Western blot analysis for the presence of the *gag* and *gag-pol* gene products. Particle production was, as expected (Figure 10), 5-10 fold higher for the codon optimised genes when compared to the wild type.

To determine the effects of the codon optimised *gag-pol* gene on vector titres, several ratios of the vector components were used. The results are shown in Figure 21. Where the *gag-pol* was the limiting component in the system (as determined by the drop in titres observed with the wild type gene), titres were 10-fold higher for the codon optimised vectors. This is in agreement with the higher protein production observed for these vectors, but suggests that under normal conditions of vector production *gag-pol* is saturating and the codon optimisation gives no maximum yield advantage.

The effect of HIV-1 *gag* INS sequences on the codon optimised gene is position dependent

It has previously been demonstrated that insertion of wild type HIV-1 *gag* sequences downstream of other RNAs, e.g. HIV-1 *tat* (56), HIV-1 *gag* (55) or CAT (57) can lead to a dramatic decrease in steady state mRNA levels, presumably as a result of the INS sequences. In other cases, e.g. for β -globin (58), it was shown that the effect was splice site dependent. Cellular AREs (AU-rich elements) that are found in the 3' UTR of labile mRNAs may confer mRNA destabilisation by inducing cytoplasmic deadenylation of the transcripts (59). To test whether HIV-1 *gag* INS sequences would destabilise the codon optimised RNA, the wild-type HIV-1 *gag* sequence, or parts of it (nt 1-625 or nt 625-1503), were amplified by PCR from the proviral clone pW13. All fragments were blunt ended and were inserted into pSYNGP or pSYNGP-RRE at either a blunted *EcoR*I or *Not*I site (upstream or downstream of the codon optimised *gag-pol*

gene repectively). As controls the wt HIV-1 *gag* in the reverse orientation (as
INS sequences have been shown to act in an orientation dependent manner, (57)
(pSYN7) and *lacZ*, excised from plasmid pCMV- β gal (CLONTECH) (in the
correct orientation) (pSYN8) were also inserted in the same site. Contrary to our
5 expectation, as shown in Figure 13, the wild type HIV-1 *gag* sequence did not
appear to significantly affect RNA or protein levels of the codon optimised gene.
We further constructed another series of plasmids (by PCR and from the same
plasmids) where the wild type HIV-1 *gag* in the sense or reverse orientation,
subfragments of *gag* (nt 1-625 or nt 625-1503), the wild type HIV-1 *gag* without
10 the ATG or with a frameshift mutation 25 bases downstream of the ATG, or nt
72-1093 of *LacZ* (excised from plasmid pH6Z), or the first 1093 bases of *lacZ*
with or without the ATG were inserted upstream of the codon optimised HIV-1
gag-pol gene in pSYNGP and/or pSYNGP-RRE (pSYN9-pSYN22, Figure 14).
Northern blot analysis showed that insertion of the wild type HIV-1 *gag* gene
15 upstream of the codon optimised HIV-1 *gag-pol* (pSYN9, pSYN10) lead to
diminished RNA levels in the presence or absence of Rev/RRE (Figure 15A,
lanes 1-4 and Figure 15B, lanes 1+12). The effect was not dependent on
translation as insertion of a wild type HIV-1 *gag* lacking the ATG or with a
frameshift mutation (pSYN12, pSYN13 and pSYN14) also diminished RNA
20 levels (Figure 15B, lanes 1-7). Western blot analysis verified that there was no
HIV-1 *gag* translation product for pSYN12-14. However, it is possible that, as the
wt HIV-1 *gag* exhibits such an adverse codon usage, it may act as a non-
translatable long 5' leader for *syngp*, and if this is the case, then the ATG
mutation should not have any effects.
25
Insertion of smaller parts of the wild type HIV-1 *gag* gene (pSYN15 and
pSYN17) also lead to a decrease in RNA levels (Figure 15B, lanes 1-3 and 8-9),
but not to levels as low as when the whole *gag* sequence was used (lanes 1-3, 4-7
and 8-9 in Figure 15B). This indicates that the effect of INS sequences is
30 dependent on their size. Insertion of the wild type HIV-1 *gag* in the reverse

orientation (pSYN11) had no effect on RNA levels (Figure 15A, lanes 1 and 5-6). However a splicing event seemed to take place in that case, as indicated by the size of the RNA (equal to the size of the codon optimised *gag-pol* RNA) and by the translation product (*gag-pol*, in equal amounts compared to pSYNGP, as verified by Western blot analysis).

These data indicate therefore that wild type HIV-1 *gag* instability sequences act in a position and size dependent manner, probably irrespective of translation. It should also be noted that the RRE was unable to rescue the destabilised RNAs through interaction with Rev.

Construction of an HIV-1 based vector system that lacks all the accessory proteins

Until now several HIV-1 based vector systems have been reported that lack all accessory proteins but Rev (49, 60). We wished to investigate whether the codon optimised gene would permit the construction of an HIV-1 based vector system that lacks all accessory proteins. We initially deleted *rev/RRE* and any residual *env* sequences, but kept the first 625 nucleotides of *gag*, as they have been shown to play a role in efficient packaging (61). Two vector genome constructs were made, pH6.1nZ (retaining only HIV sequences up to nt 625 of *gag*) and pH6.2nZ (same as pH6.1nZ, but also retaining the *env* splice acceptor). These were derived from a conventional HIV vector genome that contains RRE and expresses Rev (pH6nZ). Our 3-plasmid vector system now expressed only HIV-1 *gag-pol* and the VSV-G envelope proteins. Vector particle titres were determined as described in the previous section. A ratio of 2 : 2 : 1 of vector genome (pH6Z or pH6.1nZ or pH6.2nZ) : *gag-pol* expression vector (pGP-RRE3 or pSYNGP) : pHCMV-G was used. Transfections were performed in the presence or absence of pCMV-Rev, as *gag-pol* expression was still Rev dependent for the wild type gene. The results are summarised in Figure 22 and indicate that an HIV vector could be produced in the

total absence of Rev, but that maximum titres were compromised at 20-fold lower than could be achieved in the presence of Rev. As gag-pol expression should be the same for pSYNGP with pH6nZ or pH6.1nZ or pH6.2nZ (since it is Rev independent), as well as for pGP-RRE3 when Rev is provided *in trans*, we suspected that the vector genome retained a requirement for Rev and was therefore limiting the titres. To confirm this, Northern blot analysis was performed on cytoplasmic RNA prepared from cells transfected with pH6nZ or pH6.1nZ in the presence or absence of pCMV-Rev. As can be seen in Figure 17, lanes 1-4, the levels of cytoplasmic RNA derived from pH6nZ were 5-10 fold higher than those obtained with pH6.1nZ (compare lanes 1-2 to lanes 3-4). These data support the notion that RNA produced from the vector genome requires the Rev/RRE system to ensure high cytoplasmic levels. This may be due to inefficient nuclear export of the RNA, as INS sequences residing within gag were still present.

Further deletions in the gag sequences of the vector genome might therefore be necessary to restore titres. To date efficient packaging has been reported to require 360 (62) or 255 (63) nucleotides of gag in vectors that still retain *env* sequences, or about 40 nucleotides of gag in a particular combination of splice donor mutation, gag and *env* deletions (64, 63). In an attempt to remove the requirement for Rev/RRE in our vector genome without compromising efficient packaging we constructed a series of vectors derived from pH6nZ containing progressively larger deletions of HIV-1 sequences (only sequences upstream and within gag were retained) plus and minus a mutant major splice donor (SD) (GT to CA mutation). Vector particle titres were determined as before and the results are summarised in Figure 23. As can be seen, deletion of up to nt 360 in gag (vector pHS3nZ) resulted in an increase in titres (compared to pH6.1nZ or pH6.2nZ) and only a 5-fold decrease (titres were $1.3-1.7 \times 10^5$) compared to pH6nZ. Further deletions resulted in titres lower than pHS3nZ and similar to pH6.1nZ. In addition, the SD mutation did not have a positive effect on vector titres and in the case of pHS3nZ it resulted in a 10-fold decrease in titres (compare titres for pHS3nZ and pHS7nZ in Figure

- 23). Northern blot analysis on cytoplasmic RNA (Figure 17, lanes 1 and 5-12) showed that RNA levels were indeed higher for pH6nZ, which could account for the maximum titres observed with this vector. RNA levels were equal for pHS1nZ (lane 5), pHS2nZ (lane 6) and pHS3nZ (lane 7) whereas titres were 5-8 fold higher for pHS3nZ. It is possible that further deletions (than that found in pHS3nZ) in *gag* might result in less efficient packaging (as for HIV-1 the packaging signal extends in *gag*) and therefore even though all 3 vectors produce similar amounts of RNA only pHS3nZ retains maximum packaging efficiency. It is also interesting to note that the SD mutation resulted in increased RNA levels in the cytoplasm (compare lanes 6 and 10, 7 and 11 or 8 and 12 in Figure 17) but equal or decreased titres (Figure 23). The GT dinucleotide that was mutated is in the stem of SL2 of the packaging signal (65). It has been reported that SL2 might not be very important for HIV-1 RNA encapsidation (65, 66), whereas SL3 is of great importance (67). Folding of the wild type and SD-mutant vector sequences with the RNAdraw software program revealed that the mutation alters significantly the secondary structure of the RNA and not only of SL2. It is likely therefore that although the SD mutation enhances cytoplasmic RNA levels it does not increase titres as it alters the secondary structure of the packaging signal.
- 20 To investigate whether the titre differences that were observed with the Rev minus vectors were indeed due to Rev dependence of the genomes, the RRE sequence (nt 7769 - 8021 of the HXB2 sequence) was inserted in the *SpeI* site (downstream of the *gag* sequence and just upstream of the internal CMV promoter) of pH6.1nZ, pHS1nZ, pHS3nZ and pHS7nZ, resulting in plasmids pH6.1nZR, pHS1nZR, pHS3nZR and pHS7nZR respectively. Vector particle titres were determined with pSYNGP and pHCMVG in the presence or absence of Rev (pCMV-Rev) as before and the results are summarised in Figure 24. In the absence of Rev titres were further compromised for pH6.1nZR (7-fold compared to pH6.1nZ), pHS3nZR (6-fold compared to pHS3nZ) and pHS7nZR (2.5-fold compared to pHS7nZ). This was expected, as the RRE also acts as an instability sequence (68) and so it would

be expected to confer Rev-dependence. In the presence of Rev titres were restored to the maximum titres observed for pH6nZ in the case of pHS3nZR (5×10^5) and pH6.1nZR (2×10^5). Titres were not restored for pHS7nZR in the presence of Rev. This supports the hypothesis that the SD mutation in pHS7nZ affects the structure of the packaging signal and thus the packaging ability of this vector genome, as in this case Rev may be able to stimulate vector genome RNA levels, as for pHS3nZR and pH6.1nZR, but it can not affect the secondary structure of the packaging signal. For vector pHS1nZ inclusion of the RRE did not lead to a decrease in titres. This could be due to the fact that pHS1nZ contains only 40 nucleotides of *gag* sequences and therefore even with the RRE the size of instability sequences is not higher than for pHS2nZ that gives equal titres to pHS1nZ. Rev was able to partially restore titres for pHS1nZR (10-fold increase when compared to pHS1nZ and 8-fold lower than pH6nZ) but not fully as in the case of pHS3nZ. This is also in agreement with the hypothesis that 40 nucleotides of HIV-1 *gag* sequences might not be sufficient for efficient vector RNA packaging and this could account for the partial and not complete restoration in titres observed with pHS1nZR in the presence of Rev.

In addition, end-point titres were determined for pHS3nZ and pH6nZ with pSYNGP in HeLa and HT1080 human cell lines. In both cases titres followed the pattern observed in 293T cells, with titres being 2-3 fold lower for pHS3nZ than for pH6nZ (See Figure 10). Finally, transduction efficiency of vector produced with pHS3nZ or pH6nZ and different amounts of pSYNGP or pGP-RRE3 at different m.o.i.'s (and as high as 1) was determined in HT1080 cells. This experiment was performed as the high level gag-pol expression from pSYNGP may result in interference by genome-empty particles at high vector concentrations. As expected for VSVG pseudotyped retroviral particles (69) transduction efficiencies correlated with the m.o.i.'s, whether high or low amounts of pSYNGP were used and with pH6nZ or pHS3nZ. For m.o.i. 1 transduction efficiency was approximately 50-60% in all cases (Figure 18). The

above data indicate that no interference due to genome-empty particles is observed in this experimental system.

5 **The codon optimised *gag-pol* gene does not use the exportin-1 nuclear export pathway**

Rev mediates the export of unspliced and singly spliced HIV-1 mRNAs via the nuclear export receptor exportin-1 (CRM1) (70, 71, 72, 73, 74). Leptomycin B (LMB) has been shown to inhibit leucine-rich NES mediated nuclear export by
10 disrupting the formation of the exportin-1/NES/RanGTP complex (75, 72). In particular, LMB inhibits nucleo-cytoplasmic translocation of Rev and Rev-dependent HIV mRNAs (76). To investigate whether exportin-1 mediates the export of the codon optimised *gag-pol* constructs, the effect of LMB on protein production was tested. Western blot analysis was performed on cell lysates from
15 cells transfected with the *gag-pol* constructs (+/- pCMV-Rev) and treated or not with LMB (7.5 nM, for 20 hours, beginning treatment 5 hours post-transfection). To confirm that LMB had no global effects on transport, the expression of β -gal from the control plasmid pCMV- β Gal was also measured. An actin internal control was used to account for protein variations between samples. The results
20 are shown in Figure 16. As expected (76), the wild type *gag-pol* was not expressed in the presence of LMB (compare lanes 3 and 4), whereas LMB had no effect on protein production from the codon optimised *gag-pol*, irrespective of the presence of the RRE in the transcript and the provision of Rev *in trans* (compare lanes 5 and 6, 7 and 8, 9 and 10, 11 and 12, 5-6 and 11-12). The
25 resistance of the expression of the codon-optimised *gag-pol* to inhibition by LMB indicates that the exportin-1 pathway is not used and therefore an alternative export pathway must be used. This offers a possible explanation for the Rev independent expression. The fact that the presence of a nonfunctional Rev/RRE interaction did not affect expression implies that the RRE does not necessarily act

as an inhibitory (e.g. nuclear retention) signal *per se*, which is in agreement with previous observations (5, 58).

In conclusion, this is the first report of an HIV-1 based vector system, composed of pSYNGP, pHS3nZ and pHCMVG, where significant vector production can be achieved in the absence of all accessory proteins. These data indicate that in order to achieve maximum titres the HIV vector genome must be configured to retain efficient packaging and that this requires the retention of *gag* sequences and a splice donor. By reducing the *gag* sequence to 360 nt in pHS3nZ and combining this with pSYNGP it is possible to achieve titre of at least 10^5 I.U./ml that is only 5-fold lower than the maximum levels achieved in the presence of Rev.

Example 2 – EIAV

Codon-optimised EIAV *gag-pol* expression cassettes

We also examined if the codon-optimisation process would alter the properties of the *gag-pol* gene of the non-primate lentivirus EIAV. The sequence of the codon-optimised gene is shown from nt1103 to 5760 of SEQ ID NO:5 (Figure 9). The wild type and the codon-optimised sequences are denoted WT and CO, respectively.

The codon usage was changed to that of highly expressed mammalian genes. pESYNGP (Figure 27 and SEQ ID NO:5) was made by transferring an *XbaI*-*NotI* fragment from a plasmid containing a codon-optimised EIAV *gag/pol* gene, synthesised by Operon Technologies Inc., Alameda, CA, into pCIneo (Promega). The gene was supplied in a proprietary plasmid backbone, GeneOp. The fragment transferred to pCIneo includes sequences flanking the codon-optimised EIAV *gag/pol* ORF: **tctagaGAATTCGCCACCATG- EIAV *gag/pol*-TGAACCCGGGgcggcgc**. The ATG start and TGA stop codons are shown in bold and the recognition sequences for *XbaI* and *NotI* sites in lower case.

The expression of Gag/Pol from the codon-optimised gene was assessed with respect to that from various wild type EIAV gag/pol expression constructs by transient transfection of HEK 293T cells (Figure 25). Transfections were carried out using the calcium phosphate technique, using equal moles of each Gag/Pol expression plasmid together with a plasmid which expressed EIAV Rev either from the wild type sequence or from a codon-optimised version of the gene: pCIneoEREV (W0 99/32646) (Figure 35 and SEQ ID NO:13) or pESYNREV (Figure 36 and SEQ ID NO:14), respectively. pESYNREV is a pCIneo-based plasmid (Promega) which was made by introducing the *EcoRI* to *SalI* fragment from a synthetic EIAV REV plasmid, made by Operon Technologies Alameda, CA. The plasmid backbone was the proprietary plasmid GeneOp in which was inserted a codon-optimised EIAV REV gene flanked by *EcoRI* and *SalI* recognition sequences and a Kozak consensus sequence to drive efficient translation of the gene. The mass of DNA on each transfection was equalised by addition of pCIneo plasmid. In transfections in which a Rev expression plasmid was omitted, a similar mass of pCIneo (Promega) was used instead (lanes labelled pCIneo). Cytoplasmic extracts were prepared 48 hours post transfection and 15µg amounts of protein were fractionated by SDS-PAGE and then transferred to Hybond ECL. The Western blot was probed with a polyclonal antisera from an EIAV-infected horse and then with a secondary antibody, anti-horse horse-radish peroxidase conjugate. Development of the blot was carried out using the ECL kit (Amersham). Positive controls for the blotting and development procedure, and cytoplasmic extract from untransfected HEK 293T cells are as indicated. The positions of various EIAV proteins are indicated.

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Expression from wild type *gag/pol* was achieved from various plasmids (see Figure 25). pONY3.2T is a derivative of pONY3.1 (WO99/32646)(Figure 34 and SEQ ID NO:12) in which mutations which ablate expression of Tat and S2 have been made. In addition, the EIAV sequence is truncated downstream of the second exon of *rev*. Specifically, expression of Tat is ablated by an 83nt deletion

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in exon 2 of *tat* which corresponds with respect to the wild type EIAV sequence, Acc. No. U01866, to deletion of nt 5234-5316 inclusive. S2 ORF expression is ablated by a 51nt deletion, corresponding to nt 5346-5396 of Acc. No. U01866. The EIAV sequence is deleted downstream of a position corresponding to nt 7815 of Acc. No. U01866. These alterations do not alter *rev*, hence expression of this gene is expressed as for pONY3.1. pONY3.2 OPTI is a derivative of pONY3.1 which has the same deletions for ablation of Tat and S2 expression as described above. In addition, the first 372nt of *gag* have been 'codon-optimised' for expression in human cells. The sequence of the wild type and codon-optimised sequences present in pONY3.2OPTI in this region are compared in Figure 43. Base differences between the sequences are indicated. The region which was codon-optimised represents the region of overlap between the vector and wild-type *gag/pol* expression constructs. Reduction of homology within this region would be expected to improve the safety profile of the vector system due to the reduced chances of recombination between the vector genome and the *gag/pol* transcripts. 3.2 OPTI-Ihyg is a derivative of 3.2 OPTI in which the *SnaBI-NotI* fragment of 3.2 OPTI is transferred to pIRES1hygro (Clontech) prepared for ligation by digestion with the same sites. The *gag/pol* gene is thus placed upstream of the IRES hygromycin phosphotransferase. Of note is the fact that the resulting construct contains the intron from pCIneo, not from pIRES1hygro. pEV53B is a derivative of PEV53A (WO 98/51810) in which the EIAV-derived sequence upstream of the Gag initiation codon is reduced to include only the major splice donor and surrounding sequences: CAG/GTAAGATG, where the Gag initiation codon is shown in bold face.

The results (Figure 26) shown the Rev-dependence of Gag/Pol expression from pHORSE3.1 (WO 99/32646), which has an EIAV derived leader sequence starting just downstream of the primer binding site and an RRE placed downstream of *gag/pol* composed of the two EIAV sequences reported to have RRE activity. Expression was enhanced by the same amount when Rev

expression was driven by wild type (pCIneoERev) (Figure 35) or codon-optimised (pESYNREV) (Figure 36) genes. This result confirms the functionality of the codon-optimised Rev expression plasmid.

- 5 In contrast to expression of Gag/Pol from pONY3.1, expression from pESYNGP was not influenced by the presence of Rev, however it was slightly lower than from pONY3.1 or pON3.2T. Expression from pESYNGPRRE (Figure 30 and SEQ ID NO:7), in which the EIAV RRE sequence present in pHORSE3.1 is placed downstream of *gag/pol*, appeared slightly lower than from pESYNGP.
- 10 The levels of expression from 3.2 OPTI and 3.2OPTI-lhyg were significantly lower than from pESYNGP or pONY3.1, even in the presence of Rev. This result suggested that there may be determinants of Gag/Pol expression within the first 372nt of the *gag* and showed that 3.2 OPTI was unlikely to be useful as a basis for EIAV vector production. Furthermore it demonstrates that codon-
- 15 optimisation of only certain regions of the whole *gag/pol* gene may not lead to high levels of Rev-independent expression.

We have previously demonstrated (43) that the 5' leader (121bp upstream of the ATG start codon) and the RRE sequence (43) are important for high expression of

20 the wild type EIAV *gag-pol*. Three constructs were made that contained either the leader sequence (LpESYNGP), the leader and RRE sequences (LpESYNGPRRE) or the RRE sequence (pESYNGPRRE). The sequences of these constructs are shown in SEQ ID NOS:6-8 and Figures 28-30. They were transfected into 293T cells in either the presence or absence of Rev expression plasmid. The cell

25 supernatant was then measured for reverse transcriptase activity (RT), using a conventional RT assay, to evaluate which construct generated the highest amount of *gag-pol* mRNA. The results are shown in Figures 39 and 40. It is clear from these results that the 5' leader leads to an increase in RT activity. The ability of these Gag/Pol expression constructs to support formation of infectious vector particles

30 was also tested by transient transfection of HEK 293 cells. The results of this

analysis of show that all of the constructs could provide functional EIAV Gag/Pol, and show the Rev dependence of titre with the pONY8.0Z vector genome plasmid, which does not encode any EIAV proteins (Figure 41).

- 5 The ability of pESYNGP to act in concert with a minimal EIAV vector genome plasmid pONY8.1Z (Figure 33, SEQ ID NO:11) was evaluated (Figure 42). The result shows that the titres obtained with pESYNGP and pONY8.1Z are about 10-fold lower than from pONY3.1 and pONY8.1Z. This reduced titre reflects the lack of Rev protein in the system rather than a deficiency of Gag/Pol production which
10 we have already shown is independent of Rev expression.

Expression of EIAV Gag/Pol was also tested from pESDSYNGP (Figure 50 and SEQ ID NO:18) in which the Kozak consensus sequence of Gag is replaced by the natural EIAV splice donor. pESDSYNGP was made from pESYNGP by
15 exchange of the 306bp *EcoRI-NheI* fragment, which runs from just upstream of the start codon for gag/pol to approximately 300 base pairs inside the gag/pol ORF with a 308bp *EcoRI-NheI* fragment derived by digestion of a PCR product made using pESYNGP as template and using the following primers: SD FOR [GGCTAGAGAATTCCAGGTAAGATGGGCGATCCCCTCACCTGG] and SD
20 REV [TTGGGTACTCCTCGCTAGGTTC]. This manipulation replaces the Kozak concensus sequence upstream of the ATG in pESYNGP with the splice donor found in EIAV. The sequence between the *EcoRI* site and the ATG of gag/pol is thus CAGGTAAG, exactly as found in the natural viral sequence. Therefore the mRNA is deleted with respect to sequences upstream but not
25 downstream of the splice donor. The performance of pESDSYNGP was assessed relative to pESYNGP and other expression plasmids by measurement of reverse transcriptase activity in supernatants from transiently transfected HEK 293T cells using a Taqman-based version of the product enhanced reverse transcriptase (PERT) assay. In this method, reverse transcriptase associated with vector
30 particles is released by mild detergent treatment and used to synthesize cDNA

- using MS2 bacteriophage RNA as template. MS2 RNA template and primer are present in excess hence the amount of cDNA is proportional to the amount of RT released from the particles. Therefore, the amount of cDNA synthesised is proportional to the number of particles. MS2 cDNA is then quantitated using
- 5 Taqman technology. The assay is carried out on test samples in parallel with a vector stock of known titre and estimated particle content. The use of the standard allows creation of a 'standard curve' and allows the relative RT content of various samples to be calculated. The results of this analysis are shown in Figure 49. The results show that Gag/Pol expression is virtually identical from
- 10 pESYNGP and pESDSYNGP. The results also indicate that expression is not significantly enhanced by Rev. The activity of the Rev expression plasmid is confirmed by the result obtained with pHORSE +, in which there is an RRE downstream of the wild type EIAV *gag/pol*, and that shows a 6-fold enhancement of expression in the presence of Rev. We also noted that the expression from
- 15 pHORSE was enhanced 3-fold in the presence of Rev. Since this construct has no RRE it suggests that Rev may be having a non-specific enhancing effect on expression, possibly as a result of being expressed at high levels in this experimental system.
- 20 The ability of pESYNGP to participate in the formation of infectious viral vector particles, when co-transfected with plasmids for the vector genome and envelope was assessed by transient transfection of HEK 293T, as described previously (49, 50). Briefly, 293T cells were seeded on 6cm dishes (1.2×10^6 /dish) and 24 hours later they were transfected by the calcium phosphate procedure. The medium
- 25 was replaced 12 hours post-transfection and supernatants were harvested 48 hours post-transfection, filtered (0.45 μ m filters) and titered by transduction of D17, canine osteosarcoma cells, in the presence of 8 μ g/ml Polybrene (Sigma). Cells were seeded at 0.9×10^5 /well in 12 well plates 24 hours prior to use in titration assays. Dilutions of supernatant were made in complete media
- 30 (DMEM/10%FBS) and 0.5ml aliquots plated out onto the D17 cells. 4 hours

after addition of the vector the media was supplemented with a further 1ml of media. Transduction was assessed by X-gal staining of cells 48 hours after addition of viral dilutions.

- 5 The vector genomes used for these experiments were pONY4.0Z (Figure 31 and SEQ ID NO:9) and pONY8.0Z (Figure 32 and SEQ ID NO:10).

pONY4.0Z (WO 99/32646) was derived from pONY2.11Z by replacement of the U3 region in the 5'LTR with the cytomegalovirus immediate early promoter (pCMV). This was carried out in such a way that the first base of the transcript derived from this CMV promoter corresponds to the first base of the R region. This manipulation results in the production of high levels of vector genome in transduced cells, particularly HEK 293T cells, and has been described previously (50). pONY4.0Z expresses all EIAV proteins except for envelope, expression of
10 which is ablated by a deletion of 736nt between the *HindIII* sites present in *env*.
15

pONY8.0Z was derived from pONY4.0Z by introducing mutations which 1) prevented expression of TAT by an 83nt deletion in the exon 2 of *tat*) prevented S2 ORF expression by a 51nt deletion 3) prevented REV expression by deletion of a single base within exon 1 of *rev* and 4) prevented expression of the N-terminal portion of *gag* by insertion of T in ATG start codons, thereby changing the sequence to ATTG from ATG. With respect to the wild type EIAV sequence Acc. No. U01866 these correspond to deletion of nt 5234-5316 inclusive, nt 5346-5396 inclusive and nt 5538. The insertion of T residues was after nt 526
20 and 543.
25

The results of this analysis are shown tabulated in Figure 37, and graphically in Figure 38. Transfections were carried out with only 3 plasmids (vector genome, *gag/pol* expression plasmid and VSV-G expression plasmid) – diagonal lined
30 bars, or with four plasmids, which included the previous set of plasmids together

with an additional plasmid encoding Rev or a similar plasmid not coding a functional protein – filled bars. The result show that high titres of vector can be achieved using pESYNGP to supply EIAV Gag/Pol. The highest titres were obtained using the Rev-expressing vector genome plasmid, pONY4.0Z, and they were only slightly lower than observed when Gag/Pol was supplied by pONY3.1. Lower titres were observed with pONY8.0Z vector genome plasmid with pESYNGP than with pONY3.1. This is due to the Rev expression requirement of pONY8.0Z. Rev is expressed by pONY3.1, but not pESYNGP. These results confirm the utility of the codon-optimised Gag/Pol expression plasmid.

Use of the synthetic EIAV gag/pol gene in construction of cell lines which stably express EIAV gag/pol.

Cells lines which express high amounts of EIAV Gag/pol are required for the construction of packaging and producer cells for EIAV vectors. As a first step in their construction HEK 293 cells were stably transfected with pIRES1hyg ESYNGP (Figure 44 and SEQ ID NO:17), in which EIAV Gag/pol expression is driven by a CMV promoter, and is linked to an ORF for expression of hygromycin phosphotransferase by an EMCV IRES. pIRES1hyg ESYNGP was made as follows. The synthetic EIAV gag/pol gene and flanking sequences was transferred from pESYNGP into pIRES1hygro expression vector (Clontech). First, pESYNGP was digested with *EcoRI*, and the ends filled in by treatment with T4DNA polymerase and then digested with *NotI*. pIRES1hygro was prepared for ligation with this fragment by digestion with *NsiI*, the ends trimmed flush by treatment with T4 DNA polymerase, then digested with *NotI*. Prior to transfection into HEK 293 cells pIRES1hyg ESYNGP was digested with *AhdI* which linearises the plasmid.

Clonal cell lines were derived by serial dilution and analysed for expression of Gag/Pol by a Taqman-based product enhanced reverse transcriptase (PERT) assay. Data for the cell line Q3.29, which expressed the highest level of Gag/Pol

is shown. The analysis showed that the level of expression from the codon-optimised EIAV Gag/Pol cassette in Q3.29 was very similar to that seen for an EIAV producer line, 8Z.20, in which Gag/Pol is expressed from the pEV53B wild type expression cassette, that produced vector particles at titres of almost 10^6 transducing units per ml. (Figure 45). Assuming exponential amplification during the assay, a difference of Ct value of 1.0 corresponds to a difference of 2-fold in concentration of the reverse transcriptase released from the particles. Therefore the difference in Gag/Pol expression between Q3.29 and 8Z.20 cells is approximately 2-8 fold. Furthermore the Ct values observed indicate that the level of expression of Gag/Pol is significantly higher than in samples of pONY8G vector particles with a titre of 2×10^6 transducing units per ml on D17 cells, but made by transient transfection of HEK 293T cells. These data indicate that the codon-optimised EIAV Gag/Pol construct can be used in the construction of EIAV packaging and producer lines and confirms the previous result that expression is independent of Rev expression.

The Q3.29 cell line was then tested for its ability to support production of infectious vector particles when transfected with a vector genome plasmid, pONY8.0Z, and the VSV-G envelope expression plasmid, pRV67 and the EIAV REV expression plasmid, pESYNREV. In addition we also evaluated the performance of a plasmid pONY8.3G FB29 (-) which is modified form of the pONY8G vector genome plasmid. PONY8G is a standard EIAV vector genome used for comparison purposes. The modifications and construction of pONY8.3G FB29 (-) (SEQ ID NO:19) are described in PCT/GB00/03837 and briefly are 1) the introduction of loxP recognition sites upstream and downstream of the vector genome cassette 2) the placement of an expression cassette for codon-optimised REV, derived from pESYNREV, and driven by the FB29 U3 promoter downstream of the vector genome cassette and orientated so that the direction of transcription was towards the vector genome cassette. The REV expression cassette is located upstream of the 3' loxP site. Thus the pONY8.3G

FB29 – plasmid carries expression cassettes for the vector genome RNA and for EIAV Rev.

5 The titres were established by limiting dilution on D17 canine osteosarcoma cells and are shown in Figure 46

The titres obtained from transfections 2-6 were up to 4.5×10^6 transducing units per ml indicating levels of Gag/Pol expression sufficient to support titres at least this high. The titres obtained were not higher when additional Gag/Pol was
10 supplied (transfection 1) indicating that Gag/Pol expression was not the limitation on titre.

15 **Improved safety profile due to Gag/Pol expression from a codon-optimised expression construct**

RCR formation takes place by recombination between different components of the vector system or by recombination of vector system components with nucleotide sequences present in the producer cells. Although recombination at the DNA level during construction of producer cell lines is possible (perhaps
20 leading to insertional activation of endogenous retroelements or retroviruses) it is thought that recombination to produce RCR occurs mainly between RNA's undergoing reverse transcription, hence occurs within the mature vector particles. In consequence, recombination will be more likely to occur between RNA's which contain packaging signals, such as the vector genome and the *gag/pol*
25 mRNA. Usually however the *gag/pol* transcript is modified so that it is deleted with respect to some or all defined packaging elements, thereby reducing the chances of its involvement in recombination.

The codon-optimisation process used to create the HIV and EIAV Gag/Pol
30 expression plasmid, pSYNGP and pESYNGP, also results in disruption of sequences and structures that direct packaging as a result of introducing changes

- at approximately every 3rd nucleotide position. We have obtained evidence for the lower level of incorporation of the codon-optimised RNA derived from pESYNGP into virions.
- 5 The packaging of mRNA's derived from a wild type *gag/pol* pEV53B expression cassette, and from the codon-optimised EIAV *gag/pol* expression cassette, pESYNGP, was compared. Medium was collected from a HEK 293 based cell-lines which were stably transfected with either pEV53B (cell line B-241), or with
- 10 RNA and do not have envelopes. In some experiments, an EIAV vector genome plasmid (pECG3-CZW) was transfected into the cells to serve as an internal positive control for hybridisation and for the presence of particles capable of packaging RNA. pECG3-CZW is a derivative of pEC-LacZ (WO 98/51810) and was made from the latter by 1) reduction of *gag* sequences so that only the first
- 15 200nt of *gag*, rather than the first 577nt, was included and 2) inclusion of the woodchuck hepatitis virus post-transcriptional regulatory element (WHV PRE) (corresponding to nt 901-1800 of Acc. No. J04514) into the *NotI* site downstream of the LacZ reporter gene.
- 20 Viral particles derived from each of the cell lines were then partially purified from the medium by equilibrium density gradient centrifugation. To do this 10 ml of medium from producer cells, harvested at 24 hours after induction with sodium butyrate, was layered onto a 20-60% (w/w) sucrose gradient in TNE buffer (pH 7.4) and centrifuged for 24 hours at 25,000 rpm and 4° C in a SW28
- 25 rotor. Fractions were collected from the bottom and 10 µl of each fraction assayed for reverse transcriptase activity to locate viral particles. The results of this analysis are shown in (Figure 47) where the profile of reverse transcriptase activity is shown as a function of gradient fraction. In these figures, the top of the gradient is on the right. It should be noted that the levels of RT activity from
- 30 the pESYNGP-expressing cell were significantly lower than from pEV53B

expressing cells. To determine the RNA content of the purified virions, aliquots from the top, middle or bottom fractions were pooled (as indicated by the bars labeled T, M and B) and the RNA from each fraction was subjected to slot-blot hybridization analysis. Using a probe specific for a common region of wild type and synthetic *gag/pol*, encapsidation of RNA was easily detectable in the peak fractions (M) of virions synthesized from the wild type construct (pEV53B), but was not detected from virions synthesized from the synthetic Gag/Pol construct (pESYNGP)(Figure 48). The control for the presence of capsid capable of carrying out encapsidation was the EIAV G3-CZW vector genome which was readily detected in peak fractions from cells expressing either the wild type or synthetic *gag/pol* proteins. Even taking into account the different levels of expression from the wild type and synthetic Gag/Pol expression constructs this result indicates that the RNA from the codon-optimised *gag/pol* gene is packaged significantly less efficiently than the wild type gene and represents a significant improvement to the safety profile of the system. Of further note is that the RNA transcribed from pEV53B was packaged. This RNA is deleted with respect to sequences upstream of the splice donor sequence (CAG/GTAAG) and yet was still packaged. This points to the localisation of major packaging determinants within the *gag* coding region and is in contrast to the collected observations on the location of the packaging signal of HIV-1.

In additional experiments we have shown that the packaging of transcripts from pEV53B is only slightly lower than from pEV53A (Figure 51). This indicates further that major packaging sequences are located within the *gag* coding region. In these experiments cell line B-241 expressed pEV53B RNA and PEV-17 expressed pEV53A RNA. The EIAV vector genome used to confirm the presence of packaging competent vector particles was G3-CZR, which is the same as G3-CZW, described above, except for the replacement of the woodchuck post-transcriptional regulatory element with a sequence containing the EIAV RRE elements. Methodology was as described above.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing
5 from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology
10 or related fields are intended to be within the scope of the following claims.

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CLAIMS

1. Use of a nucleotide sequence coding for retroviral gag and pol proteins capable of assembly of a retroviral vector genome into a retroviral particle in a producer cell to generate a replication defective retrovirus in a target cell,
5 wherein the nucleotide sequence is codon optimised for expression in the producer cell.
2. Use of a nucleotide sequence coding for retroviral gag and pol proteins capable of assembly of a retroviral vector genome into a retroviral particle in a producer cell to prevent packaging of the retroviral vector genome in a target cell,
10 wherein the nucleotide sequence is codon optimised for expression in the producer cell.
3. Use of a nucleotide sequence coding for retroviral gag and pol proteins capable of assembly of a retroviral vector genome comprising at least part of a gag nucleotide sequence into a retroviral particle in a producer cell to prevent
15 recombination between said nucleotide sequence coding for retroviral gag and pol proteins and the at least part of a gag nucleotide sequence, wherein the nucleotide sequence coding for retroviral gag and pol proteins is codon optimised for expression in the producer cell.
- 20 4. A use according to any preceding claim wherein the retroviral genome further comprises a nucleotide of interest (NOI).
5. A use according to any preceding claim wherein the retroviral particle is a lentiviral particle.
6. A use according to claim 5, wherein the retroviral particle is substantially derived from HIV-1.

7. A use according to claim 6, wherein the codon optimised nucleotide sequence has the sequence shown in SEQ ID NO: 15.

8. A use according to claim 5, wherein the retroviral particle is substantially derived from EIAV.

9. A use according to claim 8, wherein the codon optimised nucleotide sequence has the sequence shown in SEQ ID NO: 16.

10. A method of producing a replication defective retrovirus comprising transfecting a producer cell with the following:

- i) a retroviral genome;
- ii) a nucleotide sequence coding for retroviral gag and pol proteins;
and
- iii) nucleotide sequences encoding other essential viral packaging components not encoded by the nucleotide sequence of (ii);

characterised in that the nucleotide sequence coding for retroviral gag and pol proteins is codon optimised for expression in the producer cell.

11. A method of preventing packaging of a retroviral genome in a target cell comprising the steps of:

a. transfecting a producer cell with the following to produce retroviral particles:

- i) a retroviral genome;
- ii) a nucleotide sequence coding for retroviral gag and pol proteins;
and
- iii) nucleotide sequences encoding other essential viral packaging components not encoded by one or more of the nucleotide sequences of (ii); and

b. transfecting a target cell with retroviral particles of step (a);

characterised in that the nucleotide sequence coding for retroviral gag and pol proteins is codon optimised for expression in the producer cell.

12. A method to prevent recombination between a retroviral vector genome and a nucleotide sequence encoding a viral polypeptide required for the assembly of the viral genome into retroviral particles comprising transfecting a producer cell with the following:

- (i) a retroviral genome comprising at least part of a gag nucleotide sequence;
 - (ii) a nucleotide sequence coding for retroviral gag and pol proteins;
- and
- (iii) nucleotide sequences encoding other essential viral packaging components not encoded by the nucleotide sequence of (ii);

characterised in that the nucleotide sequence coding for retroviral gag and pol proteins is codon optimised for expression in the producer cell.

13. A method according to any one of claims 10 to 12 wherein the retroviral genome further comprises a nucleotide of interest (NOI).

14. A method according to any one of claims 10 to 13 wherein the retroviral particle is a lentiviral particle.

15. A method according to claim 14, wherein the retroviral particle is substantially derived from HIV-1.

16. A method according to claim 15, wherein the codon optimised nucleotide sequence has the sequence shown in SEQ ID NO: 15.

17. A method according to claim 14, wherein the retroviral particle is substantially derived from EIAV.

18. A method according to claim 17, wherein the codon optimised nucleotide sequence has the sequence shown in SEQ ID NO: 16.
19. A method according to any one of claims 10 to 18, wherein iii) comprises a nucleotide sequence coding for an env protein.
20. A nucleotide sequence coding for retroviral gag and pol proteins having the sequence of SEQ. ID. No. 15 or 16.
21. A method according to any one of claims 10 to 20 wherein at least one of i) to iii) contains one or more functional accessory genes.
22. A method according to any one of claims 10 to 20 wherein i) to iii) are devoid of any functional accessory genes.
23. A viral vector system comprising:
- i) a nucleotide sequence of interest; and
 - ii) a nucleotide sequence encoding a viral polypeptide required for the assembly of viral particles wherein the nucleotide sequence is as defined in claim 20.
24. A viral production system comprising:
- i) a viral genome comprising at least one nucleotide sequence of interest; and
 - ii) a nucleotide sequence encoding a viral polypeptide required for the assembly of the viral genome into viral particles wherein the nucleotide sequence is as defined in claim 20.

25. A system according to claim 23 or claim 24 wherein the viral vector is a retroviral vector.
26. A system according to claim 25 wherein the retroviral vector is a lentiviral vector.
27. A system according to any one of claims 23 to 26 wherein the lentiviral vector is substantially derived from HIV-1 or EIAV.
28. A system according to any of claims 23 to 27 wherein the nucleotide sequence defined i-ii) also includes an envelope protein.
29. A system according to claim 28 wherein the envelope gene is codon optimised.
30. A system according to any of claims 23 to 29 wherein the nucleotide of interest is selected from a therapeutic gene, a marker gene and a selection gene.
31. A system according to any one of claims 23 to 30 comprising one or more functional accessory genes.
32. A system according to any of claims 23 to 30 devoid of any functional accessory genes.
33. A viral system according to any one of claims 23 to 32 for use in a method of producing viral particles.
34. A method for producing a viral particle which method comprises introducing into a producer cell:
- i) a viral genome as defined in any one of claims 24 to 33,

- ii) one or more nucleotide sequences as defined in claim 20 and,
- iii) nucleotide sequences encoding other essential viral packaging components not encoded by one or more of the nucleotide sequences of (ii).

35. A viral particle produced by the production system of any one of claims 24 to 33 or by the method of claim 34.

36. A viral system according to any one of claims 23 to 33, or a viral particle according to claim 35, for treating a viral infection.

37. A pharmaceutical composition comprising the viral system of any one of claims 23 to 33, or the viral particle of claim 35, together with a pharmaceutically acceptable carrier or diluent.

FIGURE 1

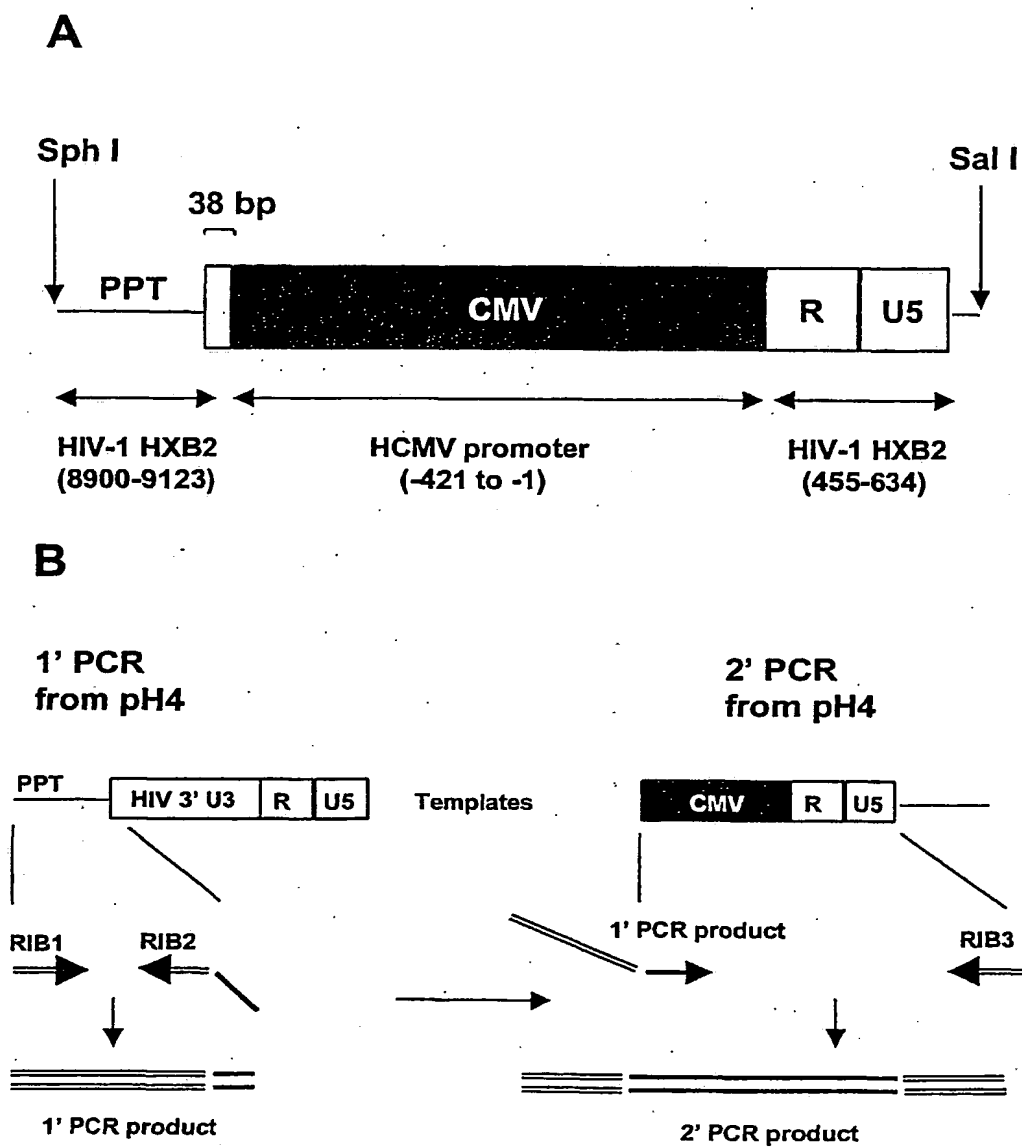


FIGURE 2

gagpol-HXB2 -> Codon Usage

DNA sequence 4308 b.p. ATGGGTGCGAGA ... GATGAGGATTAG linear

1436 codons

MW : 161929 Dalton CAI(S.c.) : 0.083 CAI(E.c.) : 0.151

TTT phe F	21	TCT ser S	3	TAT tyr Y	30	TGT cys C	18
TTC phe F	14	TCC ser S	3	TAC tyr Y	9	TGC cys C	2
TTA leu L	46	TCA ser S	19	TAA OCH Z	-	TGA OPA Z	-
TTG leu L	11	TCG ser S	1	TAG AMB Z	1	TGG trp W	37
CTT leu L	13	CCT pro P	21	CAT his H	20	CGT arg R	-
CTC leu L	7	CCC pro P	14	CAC his H	7	CGC arg R	-
CTA leu L	17	CCA pro P	41	CAA gln Q	56	CGA arg R	3
CTG leu L	16	CCG pro P	-	CAG gln Q	39	CGG arg R	3
ATT ile I	30	ACT thr T	24	AAT asn N	42	AGT ser S	18
ATC ile I	14	ACC thr T	20	AAC asn N	16	AGC ser S	16
ATA ile I	56	ACA thr T	43	AAA lys K	88	AGA arg R	45
ATG met M	29	ACG thr T	1	AAG lys K	34	AGG arg R	18
GTT val V	15	GCT ala A	17	GAT asp D	37	GGT gly G	11
GTC val V	11	GCC ala A	19	GAC asp D	26	GGC gly G	10
GTA val V	55	GCA ala A	55	GAA glu E	75	GGA gly G	61
GTG val V	15	GCG ala A	5	GAG glu E	32	GGG gly G	26

FIGURE 3a

gagpol-SYNgp [1 to 4308] -> Codon Usage

DNA sequence 4308 b.p. ATGGGCGCCCGC ... GATGAGGATTAG linear

1436 codons

MW : 161929 Dalton CAI(S.c.) : 0.080 CAI(E.c.) : 0.296

TTT phe F	5	TCT ser S	5	TAT tyr Y	10	TGT cys C	6
TTC phe F	30	TCC ser S	11	TAC tyr Y	29	TGC cys C	14
TTA leu L	2	TCA ser S	4	TAA och Z	-	TGA opa Z	-
TTG leu L	7	TCG ser S	6	TAG amb Z	1	TGG trp W	37
CTT leu L	3	CCT pro P	14	CAT his H	6	CGT arg R	2
CTC leu L	22	CCC pro P	39	CAC his H	21	CGC arg R	34
CTA leu L	6	CCA pro P	10	CAA gln Q	14	CGA arg R	3
CTG leu L	70	CCG pro P	13	CAG gln Q	81	CGG arg R	10
ATT ile I	17	ACT thr T	11	AAT asn N	13	AGT ser S	7
ATC ile I	79	ACC thr T	48	AAC asn N	45	AGC ser S	27
ATA ile I	4	ACA thr T	13	AAA lys K	25	AGA arg R	7
ATG met M	29	ACG thr T	16	AAG lys K	97	AGG arg R	13
GTT val V	5	GCT ala A	15	GAT asp D	19	GGT gly G	10
GTC val V	27	GCC ala A	56	GAC asp D	44	GGC gly G	54
GTA val V	6	GCA ala A	13	GAA glu E	29	GGA gly G	16
GTG val V	58	GCG ala A	12	GAG glu E	78	GGG gly G	28

Codon usage in human genes (MH), wild type HIV-1 Gag-pol (WT) and the codon optimised HIV-1 Gag-pol (CO)

		MH	WT	CO			MH	WT	CO			MH	WT	CO			MH	WT	CO
Ala	A	13	46	8	Cys	C	68	10	70	Leu	A	3	14	3	Ser	C	34	35	55
GC	C	53	19	65	TG	T	32	90	30	CT	C	28	8	17	AG	T	10	10	3
	G	17	11	8							G	58	14	70	TC	A	5	38	17
	T	17	24	19	Gln	A	12	53	21		T	5	11	6		C	28	10	14
					CA	G	88	47	79	TT	A	2	42	6		G	9	3	7
Arg	A	10	58	10							G	6	11	0		T	13	3	3
AG	G	18	29	11	Glu	A	25	65	38	Lys	A	18	58	28	Thr	A	14	45	16
CG	A	6	6	0	GA	G	76	35	62	AA	G	82	42	72	AC	C	57	29	52
	C	37	0	61						Phe	C	80	45	45		G	15	0	19
	G	21	6	10	Gly	A	14	53	21	TT	T	20	55	55		T	14	28	13
	T	7	0	5	GG	C	50	21	55						Tyr	C	74	20	80
Asn	C	78	29	71		G	24	24	24	Pro	A	18	52	24	TA	T	26	80	20
AA	T	22	71	29		T	12	3	0	CC	C	48	15	39					
Asp	C	75	64	70	His	C	79	30	90		G	17	3	21	Val	A	5	56	4
GA	T	25	36	30	CA	T	21	70	10		T	19	30	15	GT	C	25	8	20
																G	64	24	76
					Ile	A	5	58	8							T	7	12	0
					AT	C	18	19	92										
						T	77	23	0										

Figure 3b

FIGURE 4

env-mn [1 to 2571] -> Codon Usage

DNA sequence 2571 b.p. ATGAGAGTGAAG ... GCTTTGCTATAA linear

857 codons

MW : 97078 Dalton CAI(S.c.) : 0.083 CAI(E.c.) : 0.140

TTT phe F	13	TCT ser S	7	TAT tyr Y	15	TGT cys C	16
TTC phe F	11	TCC ser S	3	TAC tyr Y	7	TGC cys C	5
TTA leu L	20	TCA ser S	13	TAA och Z	1	TGA opa Z	-
TTG leu L	17	TCG ser S	2	TAG amb Z	-	TGG trp W	30
CTT leu L	9	CCT pro P	5	CAT his H	8	CGT arg R	-
CTC leu L	11	CCC pro P	9	CAC his H	6	CGC arg R	2
CTA leu L	12	CCA pro P	12	CAA gln Q	22	CGA arg R	1
CTG leu L	15	CCG pro P	2	CAG gln Q	19	CGG arg R	1
ATT ile I	21	ACT thr T	16	AAT asn N	50	AGT ser S	18
ATC ile I	10	ACC thr T	14	AAC asn N	13	AGC ser S	11
ATA ile I	32	ACA thr T	28	AAA lys K	32	AGA arg R	30
ATG met M	17	ACG thr T	5	AAG lys K	14	AGG arg R	15
GTT val V	8	GCT ala A	16	GAT asp D	18	GGT gly G	10
GTC val V	9	GCC ala A	7	GAC asp D	14	GGC gly G	6
GTA val V	26	GCA ala A	20	GAA glu E	36	GGA gly G	28
GTG val V	12	GCG ala A	5	GAG glu E	10	GGG gly G	12

FIGURE 5

SYNgpl60mn -> Codon Usage

DNA sequence 2571 b.p. ATGAGGGTGAAG ... GCGCTGCTGTAA linear

857 codons

MW : 97078 Dalton CAI(S.c.) : 0.074 CAI(E.c.) : 0.419

TTT phe F	-	TCT ser S	2	TAT tyr Y	1	TGT cys C	-
TTC phe F	24	TCC ser S	4	TAC tyr Y	21	TGC cys C	21
TTA leu L	-	TCA ser S	-	TAA och Z	1	TGA opa Z	-
TTG leu L	-	TCG ser S	-	TAG amb Z	-	TGG trp W	30
CTT leu L	-	CCT pro P	-	CAT his H	2	CGT arg R	1
CTC leu L	20	CCC pro P	26	CAC his H	12	CGC arg R	36
CTA leu L	1	CCA pro P	-	CAA gln Q	-	CGA arg R	-
CTG leu L	63	CCG pro P	2	CAG gln Q	41	CGG arg R	4
ATT ile I	2	ACT thr T	-	AAT asn N	2	AGT ser S	-
ATC ile I	61	ACC thr T	59	AAC asn N	61	AGC ser S	48
ATA ile I	-	ACA thr T	-	AAA lys K	1	AGA arg R	2
ATG met M	17	ACG thr T	4	AAG lys K	45	AGG arg R	6
GTT val V	-	GCT ala A	-	GAT asp D	2	GGT gly G	1
GTC val V	1	GCC ala A	40	GAC asp D	30	GGC gly G	47
GTA val V	1	GCA ala A	-	GAA glu E	3	GGA gly G	-
GTG val V	53	GCG ala A	8	GAG glu E	43	GGG gly G	8

FIGURE 6
HIV Constructs

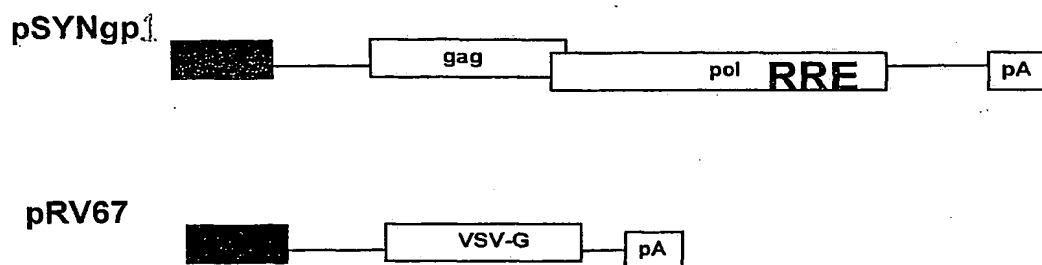
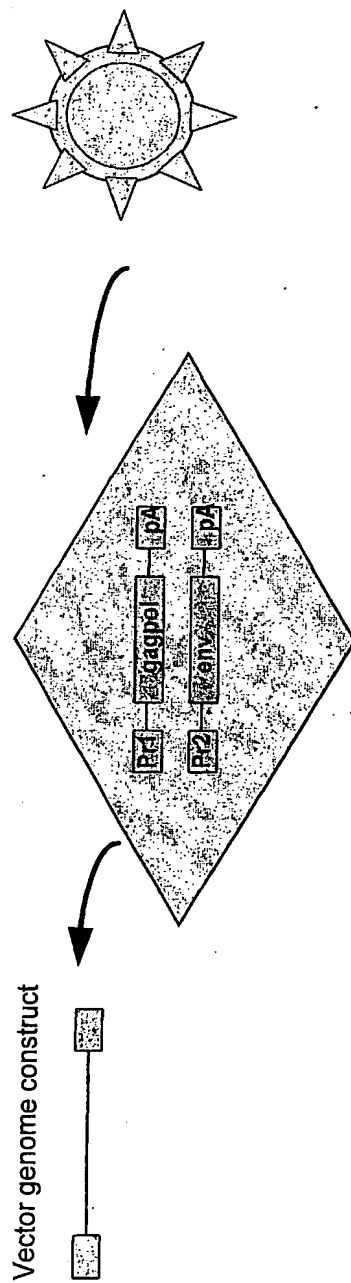


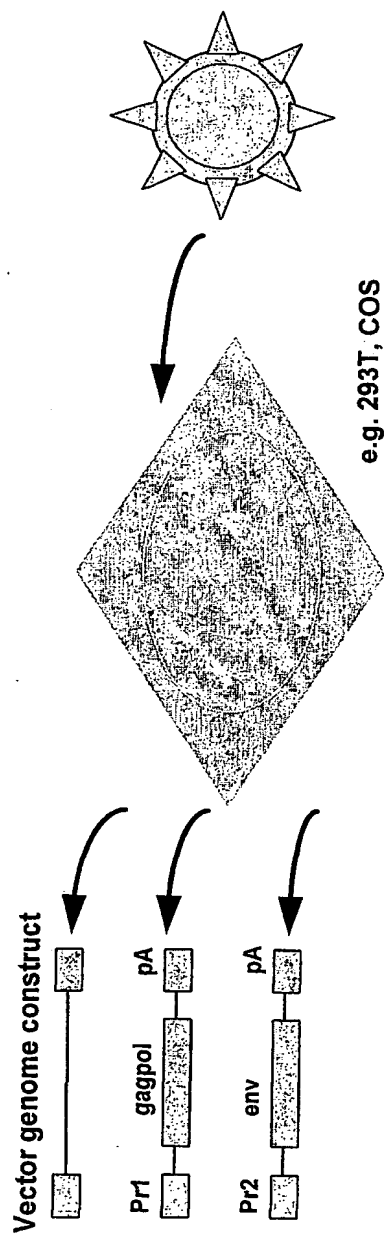
FIGURE 7

The Hit Vector System

Helper packaging cell lines



Three-plasmid cotransfection (HIT)



[illegible]

10/59

11/59

12/59

WT	ATGGGAGACCCCTTTGACATGGAGCAAGGCGCTCAAGAAGTTAGAGAAGGTGACGGTACAA	60
CO	ATGGGCGATCCCCTCACCTGGTCCAAAGCCCTGAAGAACTGGAAAAAGTCACCGTTCAG	60
WT	GGGTCTCAGAAATTAAGTACTGGTAACTGTAATTGGGCGCTAAGTCTAGTAGACTTATTT	120
CO	GGTAGCCAAAAGCTTACCACAGGCAATTGCAACTGGGCATTGTCCCTGGTGGATCTTTTC	120
WT	CATGATACCAACTTTGTAAAAGAAAAGGACTGGCAGCTGAGGGATGTCAATCCATTGCTG	180
CO	CACGACACTAATTTCTGTTAAGGAGAAAAGATTGGCAACTCAGAGACGTGATCCCCCTCTTG	180
WT	GAAGATGTAAGTCAAGACGCTGTGAGGACAAGAAAGAGAGGCCCTTTGAAAGAATGGTGG	240
CO	GAGGACGTGACCCAAACATTGTCTGGGCGAGAGCGGAAGCTTTCGAGCGCACCTGGTGG	240
WT	GCAATTTCTGCTGTAAAGATGGGCTCCAGATTAATAATGTAGTAGAGTGGAAAGGCATCA	300
CO	GCCATCAGCGCAGTCAAAATGGGGCTGCAATCAACAACGTGGTTGACGGTAAAGCTAGC	300
WT	TTCCAGCTCCTAAGAGCGAAATATGAAAAGAAGACTGCTAATAAAAAGCAGTCTGAGCCC	360
CO	TTTCAACTGTCTCGCGCTAAGTACGAGAAGAAAACCGCCAACAAGAAACAATCCGAACCT	360
WT	TCTGAAGAATATCCAATCATGATAGATGGGGCTGGAAACAGAAATTTAGACCTCTAACA	420
CO	AGCGAGGAGTACCCAATTATGATCGACGGCGCCGCAATAGGAACCTCCGCCCACTGACT	420
WT	CCTAGAGGATATACTACTTTGGGTGAATACCATACAGACAAATGGTCTATTAAATGAAGCT	480
CO	CCCAGGGGCTATACCACCTGGGTCAACACCATCCAGACAAACGGACTTTTGAACGAAGCC	480
WT	AGTCAAACTTATTTGGGATATTATCAGTAGACTGTACTTCTGAAGAAATGAATGCATTT	540
CO	TCCGAGAACCTGTTTCGGCATCTGTCTGTGGACTGCACCTCCGAAGAAATGAATGCTTTT	540
WT	TTGGATGTGGTACCTGGCCAGGCAGGACAAAAGCAGATATTACTTGATGCAATTGATAAG	600
CO	CTCGACGTGGTGCCAGGACAGGCTGGACAGAAACAGATCCTGCTCGATGCCATTGACAAG	600
WT	ATAGCAGATGATTGGGATAATAGACATCCATTACCGAATGCTCCACTGGTGGCACCACCA	660
CO	ATCGCCGACGACTGGGATAATCGCCACCCCCTGCCAAACGCCCTCTGGTGGCTCCCCCA	660
WT	CAAGGGCCTATTCCCATGACAGCAAGGTTTATTAGAGGTTTAGGAGTACCTAGAGAAAGA	720
CO	CAGGGGCTATCCCTATGACCGCTAGGTTTCATTAGGGGACTGGGGGTGCCCCGGAACGC	720
WT	CAGATGGAGCCTGCTTTTGATCAGTTTAGGCAGACATATAGACAATGGATAATAGAAGCC	780
CO	CAGATGGAGCCAGCATTTGACCAATTAGGCAGACCTACAGACAGTGGATCATCGAAGCC	780
WT	ATGTCAGAAGGCATCAAAGTGATGATTGGAAAACCTAAAGCTCAAATATTAGGCAAGGA	840
CO	ATGAGCGAGGGGATTAAAGTCATGATCGGAAGCCCAAGGCACAGAACATCAGGCAGGGG	840
WT	GCTAAGGAACCTTACCCAGAATTTGTAGACAGACTATTATCCCAAATAAAAGTGAGGGA	900
CO	GCCAAAGGAACCATACCTGAGTTTGTGACAGAGGCTTCTGTCCAGATTAAATCCGAAGGC	900
WT	CATCCACAAGAGATTTCAAATCTTGACTGATACACTGACTATTTCAGAACGCAAATGAG	960
CO	CACCTCAGGAGATCTCAAGTTCTTGACAGACACACTGACTATCCAAATGCAAATGAA	960

WT	GAATGTAGAAATGCTATGAGACATTTAAGACCAGAGGATACATTAGAAGAGAAAATGTAT	1020
CO	GAGTGCAGAAACGCCATGAGGCACCTCAGACCTGAAGATACCTGGAGGAGAAAATGTAC	1020
WT	GCTTGCAGAGACATTGGAACACAAAACAAAAGATGATGTTATTGGCAAAAGCACTTCAG	1080
CO	GCATGTCGCGACATTGGCACTACCAAGCAAAAGATGATGCTGCTCGCCAAGGCTCTGCAA	1080
WT	ACTGGTCTTGCGGGCCCATTTAAAGGTGGAGCCTTGAAAGGAGGGCCACTAAAGGCAGCA	1140
CO	ACCGGCCTGGCTGGTCCATTCAAAGGAGGAGCACTGAAGGGAGGTCCATTGAAAGCTGCA	1140
WT	CAAACATGTTATAACTGTGGGAAGCCAGGACATTTATCTAGTCAATGTAGAGCACCTAAA	1200
CO	CAAACATGTTATAATTGTGGGAAGCCAGGACATTTATCTAGTCAATGTAGAGCACCTAAA	1200
WT	GTCTGTTTTAAATGTAAACAGCCTGGACATTTCTCAAAGCAATGCAGAAGTGTTCAAAA	1260
CO	GTCTGTTTTAAATGTAAACAGCCTGGACATTTCTCAAAGCAATGCAGAAGTGTTCAAAA	1260
WT	AACGGGAAGCAAGGGGCTCAAGGGAGGCCCCAGAAACAAACTTCCCGATACAACAGAAG	1320
CO	AACGGGAAGCAAGGGGCTCAAGGGAGGCCCCAGAAACAAACTTCCCGATACAACAGAAG	1320
WT	AGTCAGCACAAACAATCTGTTGTACAAGAGACTCCTCAGACTCAAAATCTGTACCCAGAT	1380
CO	AGTCAGCACAAACAATCTGTTGTACAAGAGACTCCTCAGACTCAAAATCTGTACCCAGAT	1380
WT	CTGAGCGAAATAAAAAAGGAATACAATGTCAAGGAGAAGGATCAAGTAGAGGATCTCAAC	1440
CO	CTGAGCGAAATAAAAAAGGAATACAATGTCAAGGAGAAGGATCAAGTAGAGGATCTCAAC	1440
WT	CTGGACAGTTTGTGGGAGTAACATATAATCTAGAGAAAAGGCCCTACTACAATAGTATTAA	1500
CO	CTGGACAGTTTGTGGGAGTAACATATAATCTAGAGAAAAGGCCCTACTACAATAGTATTAA	1500
WT	TTAATGATACTCCCTTAAATGTACTGTTAGACACAGGAGCAGATACTTCAGTGTTGACTA	1560
CO	TCAATGACACCCCTCTTAAATGTCTGCTGGACACCGGAGCCGACACCAGCGTTCTCACTA	1560
WT	CTGCACATTATAATAGGTTAAAAATATAGAGGGAGAAAATATCAAGGGACGGGAATAATAG	1620
CO	CTGCTCACTATAACAGACTGAAATACAGAGGAAGGAAATACCAGGGCACAGGCATCATCG	1620
WT	GAGTGGGAGGAAATGTGGAACATTTTCTACGCCTGTGACTATAAAGAAAAAGGGTAGAC	1680
CO	GCGTTGGAGGCAACGTCGAAACCTTTTCCACTCCTGTCAACCATCAAAAAGAGGGGAGAC	1680
WT	ACATTAAAGACAAGAATGCTAGTGGCAGATATTCAGTGACTATTTTGGGACGAGATATTC	1740
CO	ACATTAAACCCAGAATGCTGGTCGCCGACATCCCGTCACCATCCTTGGCAGAGACATTC	1740
WT	TTCAGGACTTAGGTGCAAAATTGGTTTTGGCACAGCTCTCCAAGGAAATAAAATTTAGAA	1800
CO	TCCAGGACCTGGGCGCTAAACTCGTGTGTCGACAACTGTCTAAGGAAATCAAGTTCCGCA	1800
WT	AAATAGAGTTAAAGAGGGGCACAATGGGGCCAAAAATTCCTCAATGGCCACTCACTAAGG	1860
CO	AGATCGAGCTGAAAGAGGGGCACAATGGGTCCAAAAATCCCCAGTGGCCCCCTGACCAAG	1860
WT	AGAACTAGAAAGGGGCCAAAGAGATAGTCCAAAGACTATTGTGAGAGGGAAAAATATCAG	1920
CO	AGAAGCTTGAGGGCGCTAAGGAAATCGTGCAGCGCCTGCTTTCTGAGGGCAAGATTAGCG	1920
WT	AAGCTAGTGACAATAATCCTTATAATTACCCATATTTGTAATAAAAAAGAGGTCTGGCA	1980
CO	AGGCCAGCGACAATAACCTTTACAACAGCCCCATCTTGTGATTAAAGAAAGGAGCGGCA	1980

WT	AATGGAGGTTATTACAAGATCTGAGAGAATTAAACAAAACAGTACAAGTAGGAACCGAAA	2040
CO	AATGGAGACTCCTGCAGGACCTGAGGGAACCTCAACAAGACCGTCCAGGTCGGAAC TGAGA	2040
WT	TATCCAGAGGATTGCCTCACCCGGGAGGATTAATTAATGTAAACACATGACTGTATTAG	2100
CO	TCTCTCGCGGACTGCCTCACCCCGGGCGGCTGATTAAATGCAAGCACATGACAGTCCTTG	2100
WT	ATATTGGAGATGCATATTTCACTATACCCCTTAGATCCAGAGTTTAGACCATATACAGCTT	2160
CO	ACATTGGAGACGCTTATTTTACCATCCCCCTCGATCCTGAATTTGCCCCCTATACTGCTT	2160
WT	TCACTATTCCCTCCATTAAATCATCAAGAACCAGATAAAAGATATGTGTGGAAATGTTTAC	2220
CO	TTACCATCCCCAGCATCAATCACCAGGAGCCCGATAAACGCTATGTGTGGAAGTGCTCC	2220
WT	CACAAGGATTTCGTGTTGAGCCCATATATATATATCAGAAAACATTACAGGAAATTTTACAAC	2280
CO	CCCAGGATTGTGCTTAGCCCCCTACATTTACCAGAAGACACTTCAAGAGATCCTCCAAC	2280
WT	CTTTTAGGGAAAGATATCCTGAAGTACAATTGTATCAATATATGGATGATTGTTCATGG	2340
CO	CTTTCGCGAAAGATACCCAGAGGTTCAACTCTACCAATATATGGACGACCTGTTTCATGG	2340
WT	GAAGTAATGGTTCTAAAAACAACACAAAGAGTTAATCATAGAATTAAGGGCGATCTTAC	2400
CO	GGTCCAACGGGTCTAAGAAGCAGCACAAGGAACCTCATCATCGAAGTGAAGGCAATCCTCC	2400
WT	TGGAAGGGGTTTGTGAGACACCAGATGATAAATTACAAGAAGTGCCACCTTATAGCTGGC	2460
CO	TGGAGAAAGGCTTCGAGACACCCGACGACAAGCTGCAAGAAGTTCCTCCATATAGCTGGC	2460
WT	TAGGTTATCAACTTTGTCTGAAAATTGGAAAGTACAAAAAATGCAATTAGACATGGTAA	2520
CO	TGGGCTACCAGCTTTGCCCTGAAAAGTGGAAAGTCCAGAAGATGCAGTTGGATATGGTCA	2520
WT	AGAATCCAACCCTTAATGATGTGCAAAAATTAATGGGGAATATAACATGGATGAGCTCAG	2580
CO	AGAACCCAACACTGAACGACGTCCAGAAGCTCATGGGCAATATTACCTGGATGAGCTCCG	2580
WT	GGATCCCAGGGTTGACAGTAAACACATTGCAGCTACTACTAAGGGATGTTTAGAGTTGA	2640
CO	GAATCCCTGGGCTTACCGTTAAGCACATTGCGCGCAACTACAAAAGGATGCCTGGAGTTGA	2640
WT	ATCAAAAAGTAATTTGGACGGAAGAGGCACAAAAGAGTTAGAAGAAAATAATGAGAAGA	2700
CO	ACCAGAAGGTCATTTGGACAGAGGAAGCTCAGAAGGAAGTGGAGGAGAATAATGAAAAGA	2700
WT	TTAAAAATGCTCAAGGGTTACAATATTATAATCCAGAAGAAGAAATGTTATGTGAGGTTG	2760
CO	TTAAGAATGCTCAAGGGCTCCAATACTACAATCCGAAGAAGAAATGTTGTGCGAGGTCG	2760
WT	AAATTACAAAAAATTATGAGGCAACTTATGTTATAAAACAATCACAAGGAATCCTATGGG	2820
CO	AAATCACTAAGAACTACGAAGCCACCTATGTCATCAACAGTCCCAAGGCATCTGTGCGG	2820
WT	CAGGTAAAAAGATTATGAAGGCTAATAAGGGATGGTCAACAGTAAAAAATTTAATGTTAT	2880
CO	CCGGAAGAAAATCATGAAGGCCAACAAAGGCTGGTCCACCGTTAAAAATCTGTATGCTCC	2880
WT	TGTTGCAACATGTGGCAACAGAAAGTATTACTAGAGTAGGAAAATGTCCAACGTTTAAGG	2940
CO	TGCTCCAGCACGTGCGCCACCGAGTCTATCACC CGCTCGGCAAGTGCCCCACCTTCAAAG	2940
WT	TACCATTTACCAAAGAGCAAGTAATGTGGGAAATGCAAAAAGGATGGTATTATCTTGGC	3000
CO	TTCCCTTCACTAAGGAGCAGGTGATGTGGGAGATGCAAAAAGGCTGGTACTACTCTTGGC	3000

WT	TCCCAGAAATAGTATATACACATCAAGTAGTTCATGATGATTGGAGAATGAAATTGGTAG	3060
CO	TTCCCGAGATCGTCTACACCCACCAAGTGGTGCACGACGACTGGAGAATGAAGCTTGTCTG	3060
WT	AAGAACCTACATCAGGAATAACAATATACACTGATGGGGGAAAACAAAATGGAGAAGGAA	3120
CO	AGGAGCCCACTAGCGGAATTACAATCTATACCGACGGCGGAAAGCAAACGGAGAGGGAA	3120
WT	TAGCAGCTTATGTGACCAGTAATGGGAGAACTAAACAGAAAAGGTTAGGACCTGTCACTC	3180
CO	TCGCTGCATACGTACATCTAACGGCCGCACCAAGCAAAGAGGCTCGGCCCTGTCACTC	3180
WT	ATCAAGTTGCTGAAAGAATGGCAATACAAATGGCATTAGAGGATACCAGAGATAAACCAAG	3240
CO	ACCAGGTGGCTGAGAGGATGGCTATCCAGATGGCCCTTGAGGACACTAGAGACAAGCAGG	3240
WT	TAAATATAGTAACTGATAGTTATTATTGTTGGAAAAATATTACAGAAGGATTAGGTTTAG	3300
CO	TGAACATTGTGACTGACAGCTACTACTGCTGGAAAAACATCACAGAGGGCCTTGCCCTGG	3300
WT	AAGGACCACAAAGTCCTTGGTGGCCTATAATACAAAATATACGAGAAAAAGAGATAGTTT	3360
CO	AGGGACCCCACTCTCCCTGGTGGCCTATCATCCAGAATATCCGCGAAAAGGAAATTGTCT	3360
WT	ATTTTGCTTGGGTACCTGGTCACAAAGGGATATATGGTAATCAATTGGCAGATGAAGCCG	3420
CO	ATTTGCGCTGGGTGCCTGGACACAAAGGAATTACGGCAACCAACTCGCCGATGAAGCCG	3420
WT	CAAAAATAAAGAAGAAATCATGCTAGCATACCAAGGCACACAAATTAAAGAGAAAAGAG	3480
CO	CCAAAATAAAGAGGAAATCATGCTTGCCTACCAGGGCACACAGATTAAGGAGAAGAGAG	3480
WT	ATGAAGATGCAGGGTTTGACTTATGTGTTTCTTATGACATCATGATACCTGTATCTGACA	3540
CO	ACGAGGACGCTGGCTTTGACCTGTGTGTGCCATACGACATCATGATTCCCGTTAGCGACA	3540
WT	CAAAAATCATACCCACAGATGTAAAAATTCAAGTTCCTCCTAATAGCTTTGGATGGGTCA	3600
CO	CAAAGATCATTCCAACCGATGTCAAGATCCAGGTGCCACCCAATTCAATTTGGTTGGGTGA	3600
WT	CTGGGAAATCATCAATGGCAAAACAGGGGTTATTAATTAATGGAGGAATAATTGATGAAG	3660
CO	CCGGAAGTCCAGCATGGCTAAGCAGGGTCTTCTGATTAACGGGGGAATCATTGATGAAG	3660
WT	GATATACAGGAGAAATACAAGTGATGTACTAATATTGGAAAAAGTAATATTAAATTAA	3720
CO	GATACACCGGCGAAATCCAGGTGATCTGCACAAATATCGGCAAAAGCAATATTAAAGCTTA	3720
WT	TAGAGGGACAAAAATTTGCACAATTAAATTATACTACAGCATCACTCAAATTCAGACAGC	3780
CO	TCGAAGGGCAGAAGTTGCTCAACTCATCATCTCCAGCACCACAGCAATTCAGACAAC	3780
WT	CTTGGGATGAAAAATAAAATATCTCAGAGAGGGGATAAAGGATTGGAAGTACAGGAGTAT	3840
CO	CTTGGGACGAAAACAAGATTAGCCAGAGAGGTGACAAGGGCTTCGGCAGCACAGGTGTGT	3840
WT	TCTGGGTAGAAAAATATTAGGAAGCACAGATGAACATGAGAATTGGCATAACATCACCAA	3900
CO	TCTGGGTGGAGAACATCCAGGAAGCACAGGACGAGCAGAGAATTGGCACACCTCCCTTA	3900
WT	AGATATTGGCAAGAAATTATAAGATACCATTTGACTGTAGCAAAACAGATAACTCAAGAAT	3960
CO	AGATTTTGGCCCCGAATTACAAGATCCCCTGACTGTGGCTAAGCAGATCACACAGGAAT	3960
WT	GTCCCTCATTGCACTAAGCAAGGATCAGGACCTGCAGGTTGTGTGATGAGATCTCCTAATC	4020
CO	GCCCCCACTGCACCAACAAGGTTCTGGCCCCGCGGCTGGGTGATGAGGTCCCCCAATC	4020

WT	ATTGGCAGGCAGATTGCACACATTTGGACAATAAGATAATATTGACTTTTGTAGAGTCAA	4080
CO	ACTGGCAGGCAGATTGCACCCACCTCGACAACAAAATTATCCTGACCTTCGTGGAGAGCA	4080
WT	ATTCAGGATACATACATGCTACATTATTGTCAAAGAAAATGCATTATGTACTTCATTGG	4140
CO	ATTCGGGTACATCCACGCAACACTCCTCTCCAAGGAAAATGCATTGTGCACCTCCCTCG	4140
WT	CTATTTTAGAATGGGCAAGATTGTTTTACCAAAGTCCTTACACACAGATAACGGCACTA	4200
CO	CAATTCCTGGAATGGGCCAGGCTGTTCTCTCCAAAATCCCTGCACACCGACAACGGCACCA	4200
WT	ATTTTGTGGCAGAACCAGTTGTAAATTTGTGTAAGTTCCTAAAGATAGCACATACCACAG	4260
CO	ACTTTGTGGCTGAACCTGTGGTGAATCTGCTGAAGTTCCTGAAAATCGCCACACCACTG	4260
WT	GAATACCATATCATCCAGAAAGTCAGGGTATTGTAGAAAGGGCAAATAGGACCTTGAAAG	4320
CO	GCATTCCCTATCACCTGAAAGCCAGGGCATTGTGAGAGGGCCACAGAACTCTGAAAG	4320
WT	AGAAGATTCAAAGTCATAGAGACAACACTCAAACACTGGAGGCAGCTTTACAACTTGCTC	4380
CO	AAAAGATCCAATCTCACAGAGACAATACACAGACATTGGAGGCCGCACTTCAGCTCGCCC	4380
WT	TCATTACTTGTAACAAAGGGAGGGAAGTATGGGAGGACAGACACCATGGGAAGTATTTA	4440
CO	TTATCACCTGCAACAAAGGAAGAGAAAGCATGGGCGGCCAGACCCCTGGGAGGTCTTCA	4440
WT	TCACTAATCAAGCACAAAGTAATACATGAGAACTTTTACTACAGCAAGCACAACTCTCCA	4500
CO	TCACTAACCAGGCCAGGTCATCCATGAAAAGCTGCTCTTGCAGCAGGCCAGTCTCTCCA	4500
WT	AAAAATTTGTTTTTACAAAATCCCTGGTGAACATGATTGGAAGGGACCTACTAGGGTGC	4560
CO	AAAAGTTCTGCTTTTATAAGATCCCCGGTGAGCACGACTGGAAGGTCTTACAAGAGTTT	4560
WT	TGTGGAAGGGTGATGGTGCACTAGTAGTTAATGATGAAGGAAAGGGAATAATTGCTGTAC	4620
CO	TGTGGAAGGAGACGGCGCAGTTGTGGTGAACGATGAGGGCAAGGGGATCATCGCTGTGC	4620
WT	CATTAACCAGGACTAAGTTACTAATAAAACCAAATTGA	4658
CO	CCCTGACACGCACCAAGCTTCTCATCAAGCCAACTGA	4658

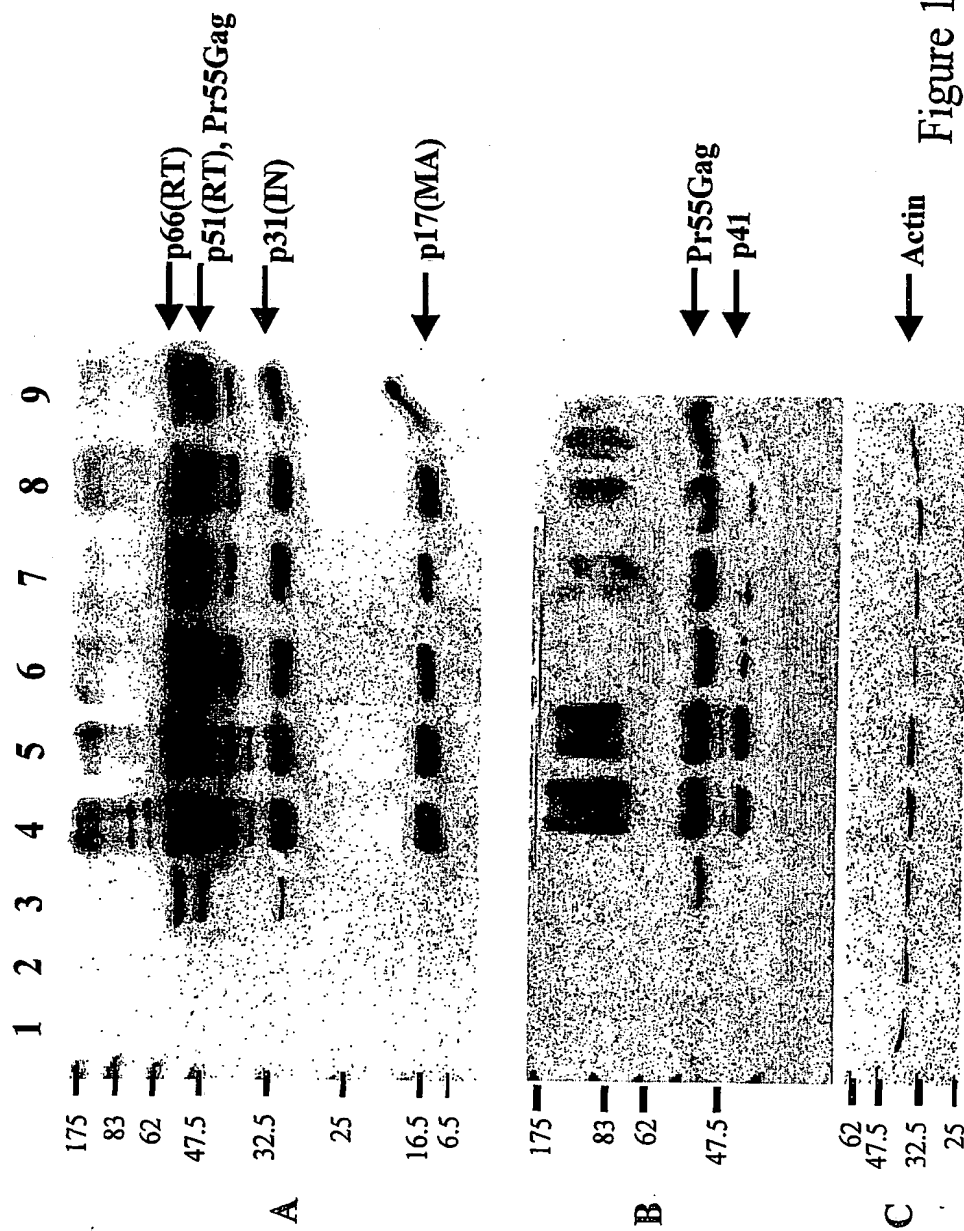


Figure 10

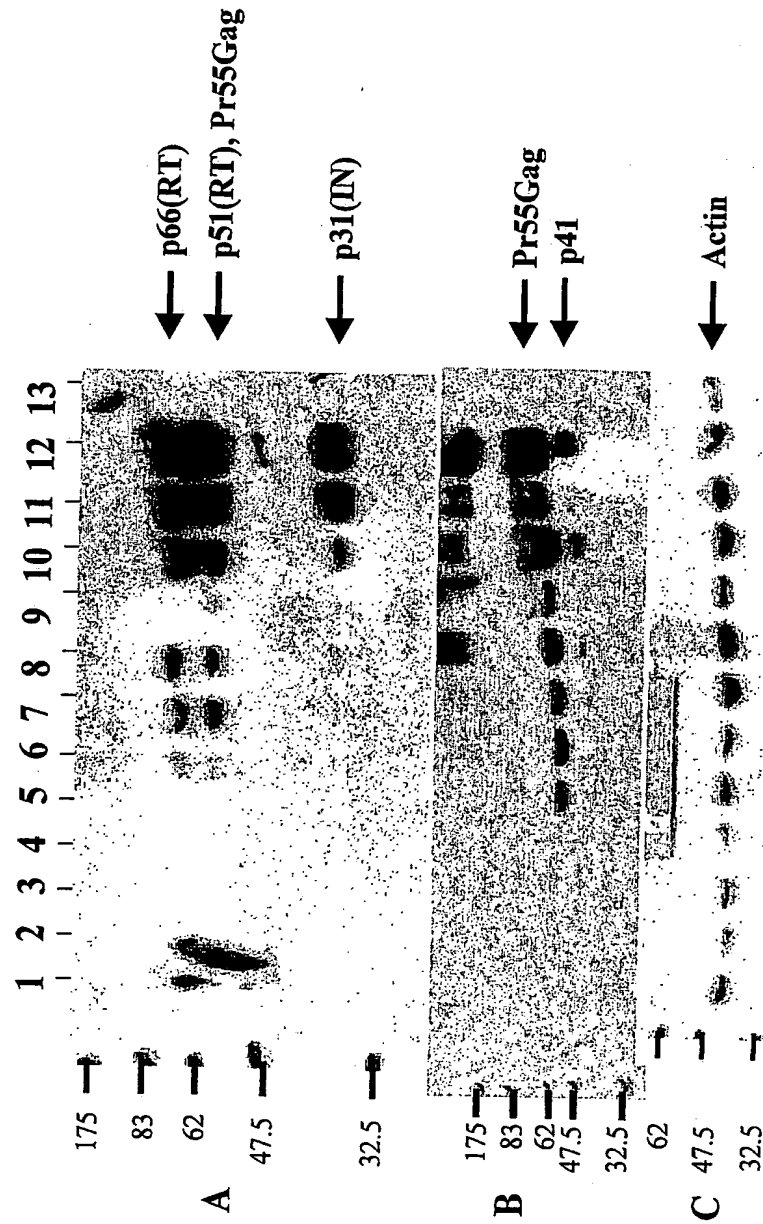


Figure 11

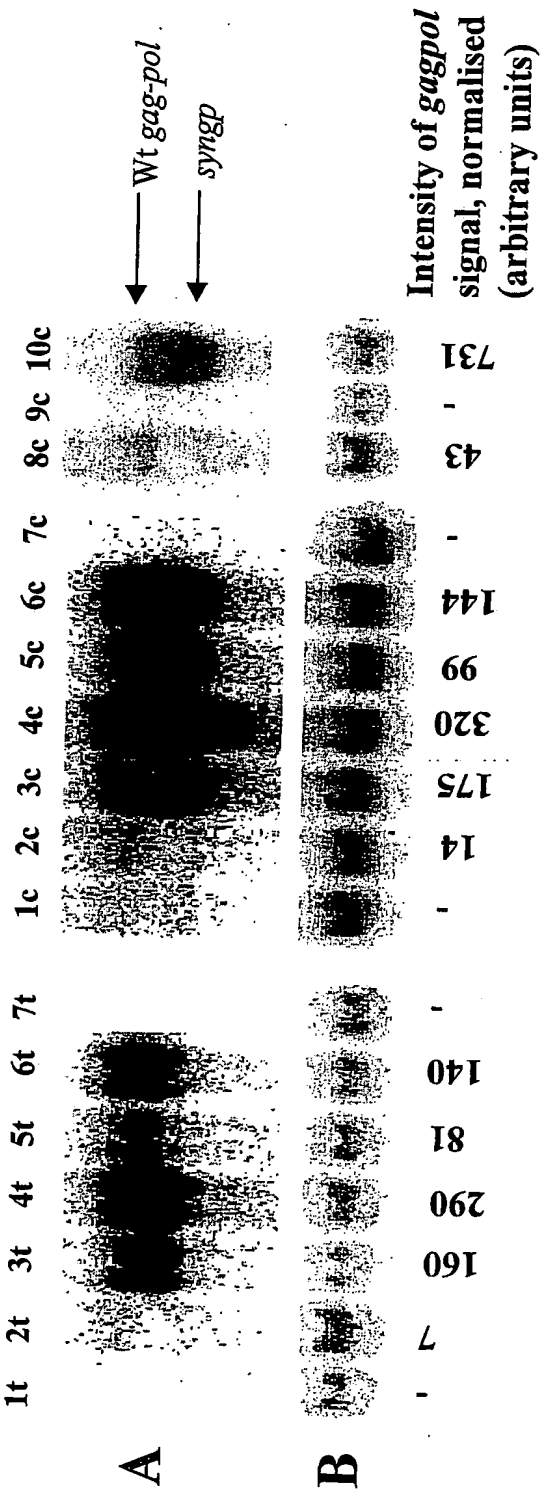


Figure 12

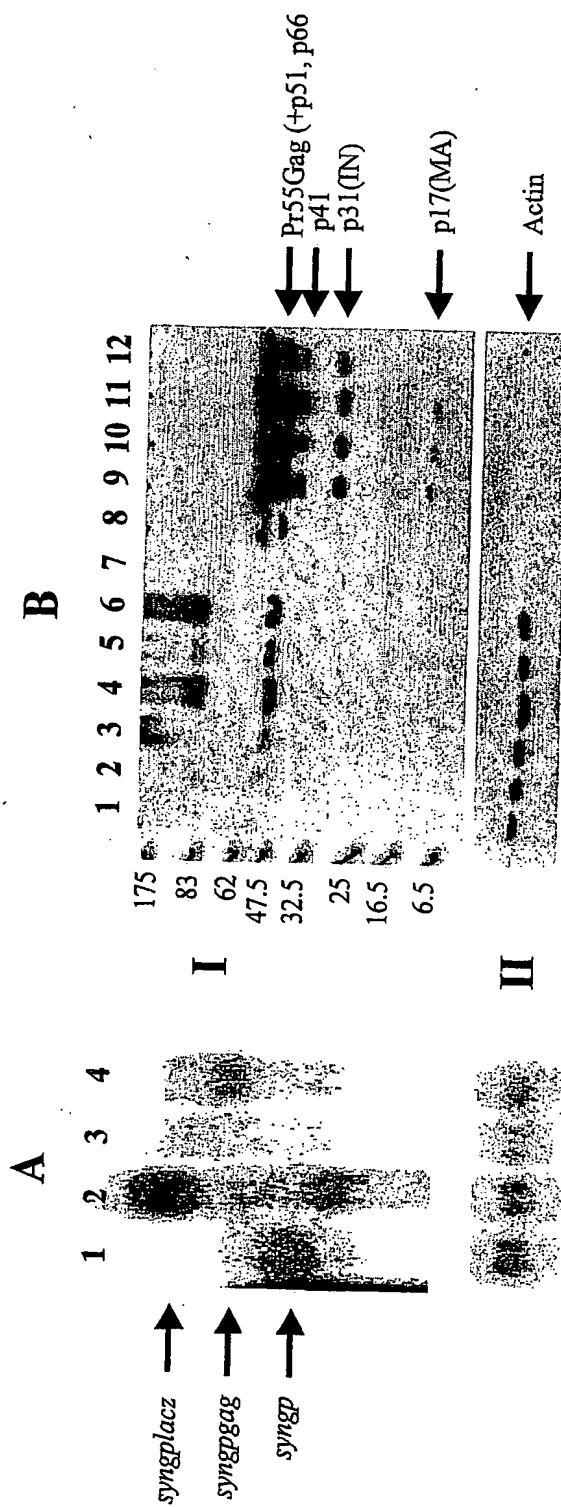


Figure 13

pSYN6 CMVp—Syn *gp*—HXB2 *gag*—pA
 pSYN7 CMVp—Syn *gp*—HXB2 *gag,r*—pA
 pSYN8 CMVp—Syn *gp*—*Lac Z*—pA
 pSYN9 CMVp—HXB2 *gag*—Syn *gp*—pA
 pSYN10 CMVp—HXB2 *gag*—Syn *gp*—RRE—pA
 pSYN11 CMVp—HXB2 *gag,r*—Syn *gp*—RRE—pA
 pSYN12 CMVp—HXB2 *gag-ΔATG*—Syn *gp*—pA
 pSYN13 CMVp—HXB2 *gag-fr.sh.*—Syn *gp*—pA
 pSYN14 CMVp—HXB2 *gag-ΔATG*—Syn *gp*—RRE—pA
 pSYN15 CMVp—HXB2 *gag 625-1503*—Syn *gp*—pA
 pSYN17 CMVp—HXB2 *gag 1-625*—Syn *gp*—pA

Figure 14

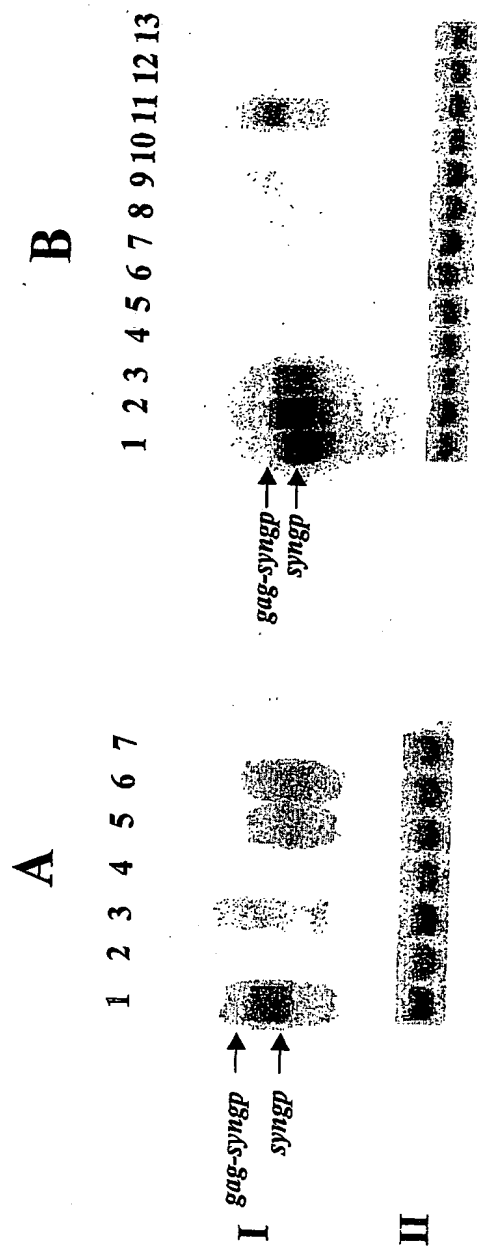


Figure 15

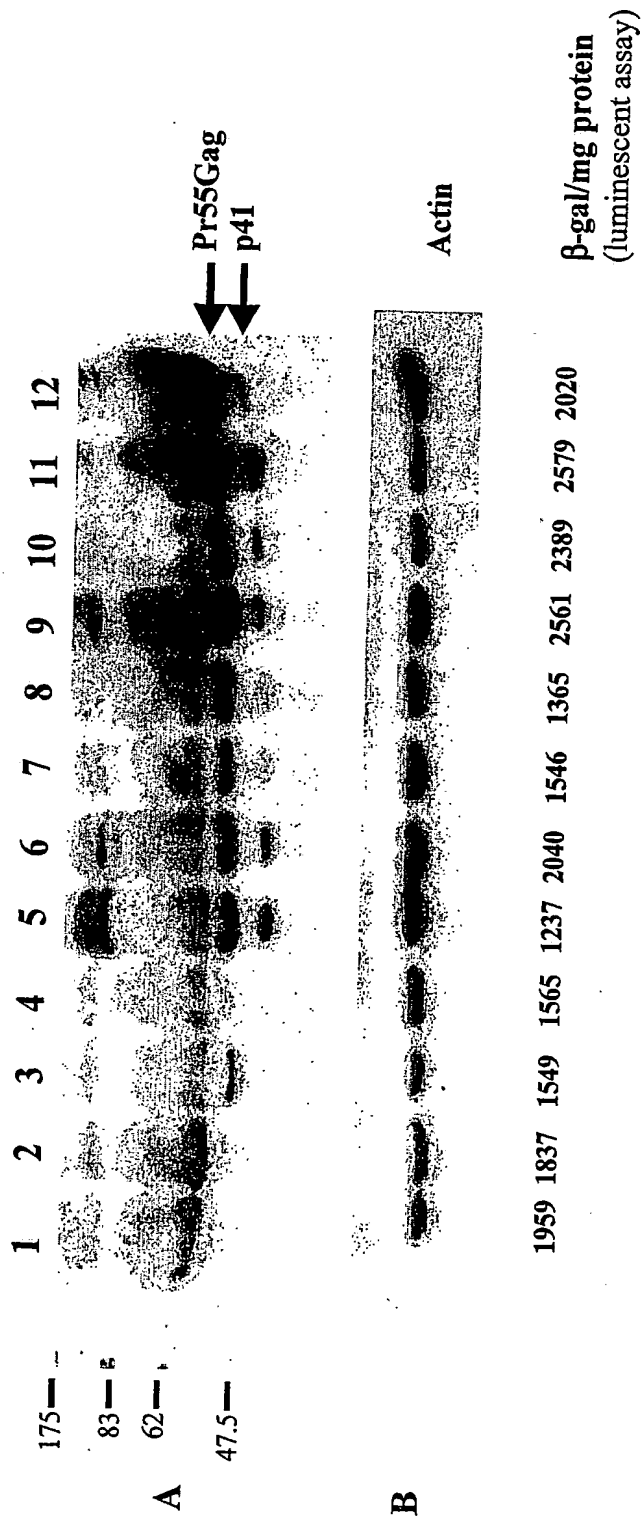


Figure 16

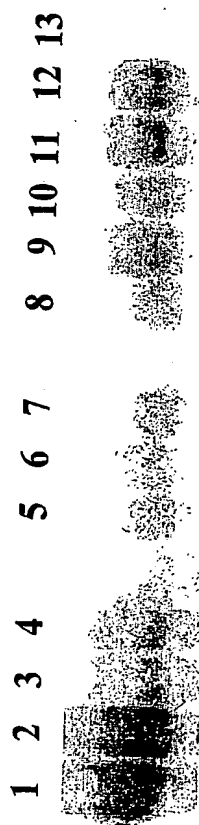
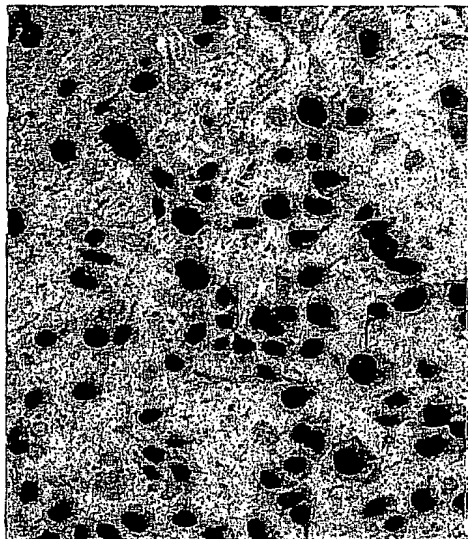
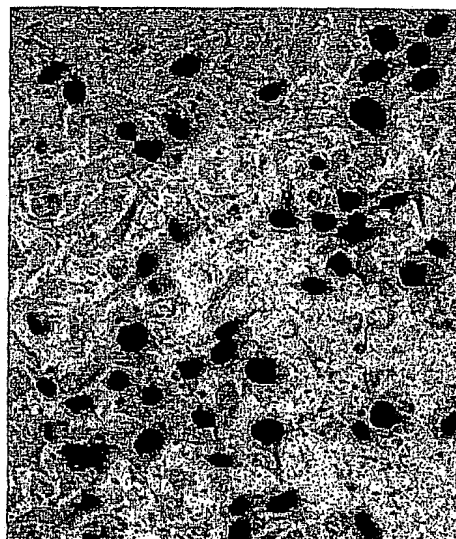


Figure 17

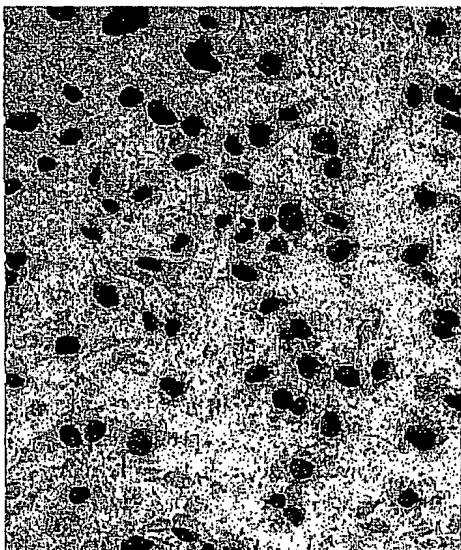
B. pH6nZ (15µg) + pSYNGP (0.5µg)



D. pH6nZ (15µg) + pSYNGP (0.5µg)



A. pH6nZ (15µg) + pSYNGP (5µg)



C. pH6nZ (15µg) + pGP-RRE3 (5µg)

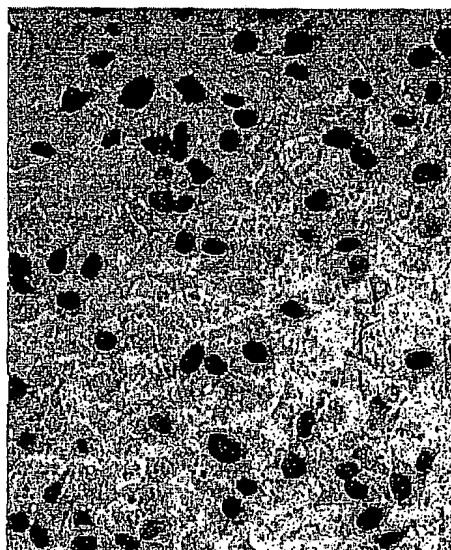


Figure 18

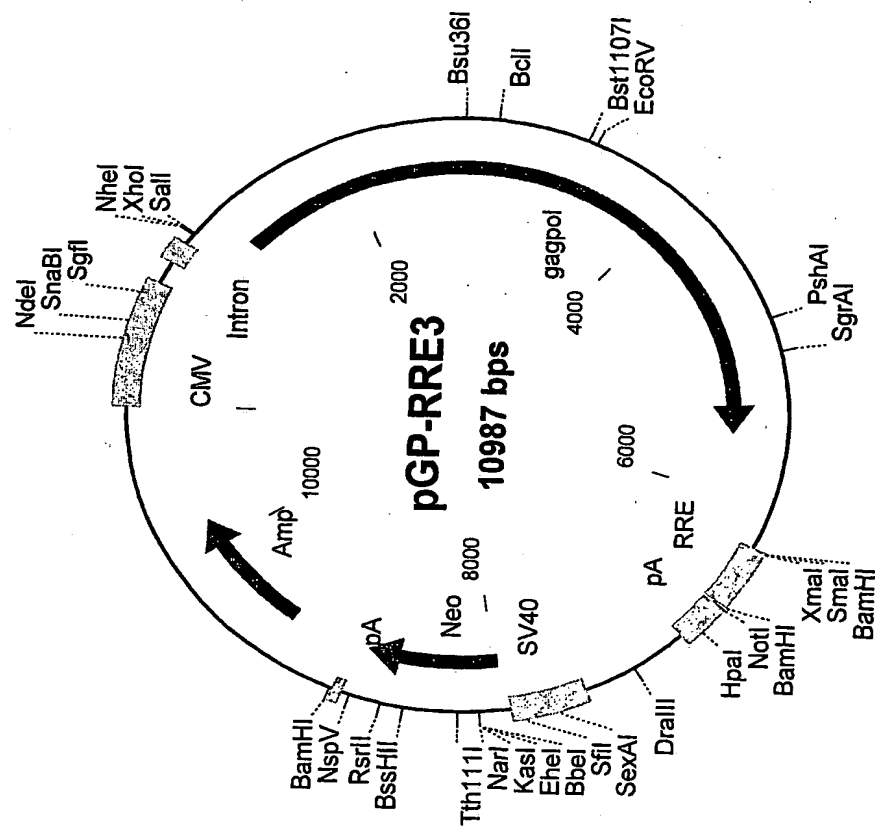


Figure 19

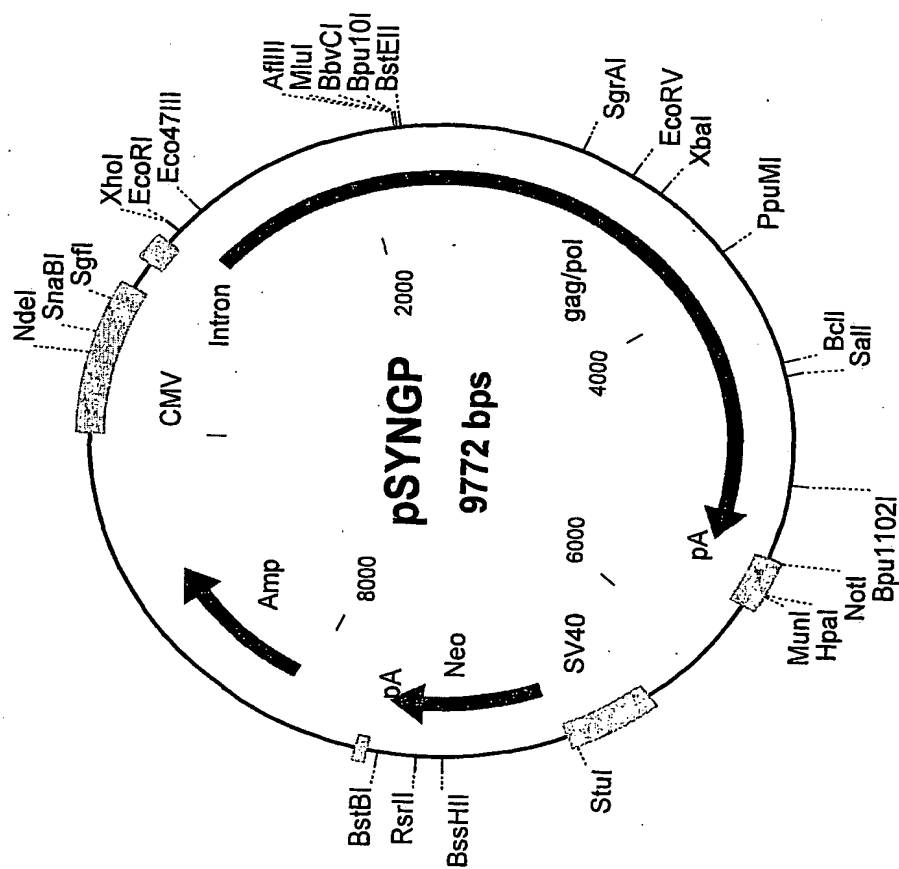


Figure 20

FIGURE 21

Amount of pfl6nZ (μ g)	Ratio of gagpol expression plasmid to pfl6nZ (μ g/ μ g)	Titre, pGP-RRE3	Titre, pSYNGP	Titre, pSYNGP-RRE	Titre, pSYNGP-ERR
5	1:1	6×10^5	5×10^5	4×10^5	3×10^5
12.5	1:2.5	8×10^5	6.5×10^5		
10	1:10	3×10^5	4×10^5		
5	1:20	3.5×10^5	2.5×10^5		
12.5	1:50	3.5×10^4	3×10^5	3×10^5	3×10^5
18	1:180	2.5×10^4	3×10^5		

Figure 22

Gag-pol expression vector	Vector genome	Rev expression	Titre (IU/ml) ^a
pGP-RRE3	pH6nZ	Yes / from pH6Z	8.0±0.2 x 10 ⁵
pGP-RRE3	pH6.1nZ	No	2.1±0.3 x 10 ³
pGP-RRE3	pH6.1nZ	Yes / from pCMV-Rev	4.1±0.3 x 10 ⁴
pGP-RRE3	pH6.2nZ	No	1.7±0.4 x 10 ³
pGP-RRE3	pH6.2nZ	Yes / from pCMV-Rev	5.6±0.3 x 10 ⁴
pSYNGP	pH6nZ	Yes / from pH6Z	7.8±0.2 x 10 ⁵
pSYNGP	pH6.1nZ	No	5.4±0.3 x 10 ⁴
pSYNGP	pH6.1nZ	Yes / from pCMV-Rev	5.2±0.4 x 10 ⁴
pSYNGP	pH6.2nZ	No	5.0±0.2 x 10 ⁴
pSYNGP	pH6.2nZ	Yes / from pCMV-Rev	5.5±0.3 x 10 ⁴

Figure 23

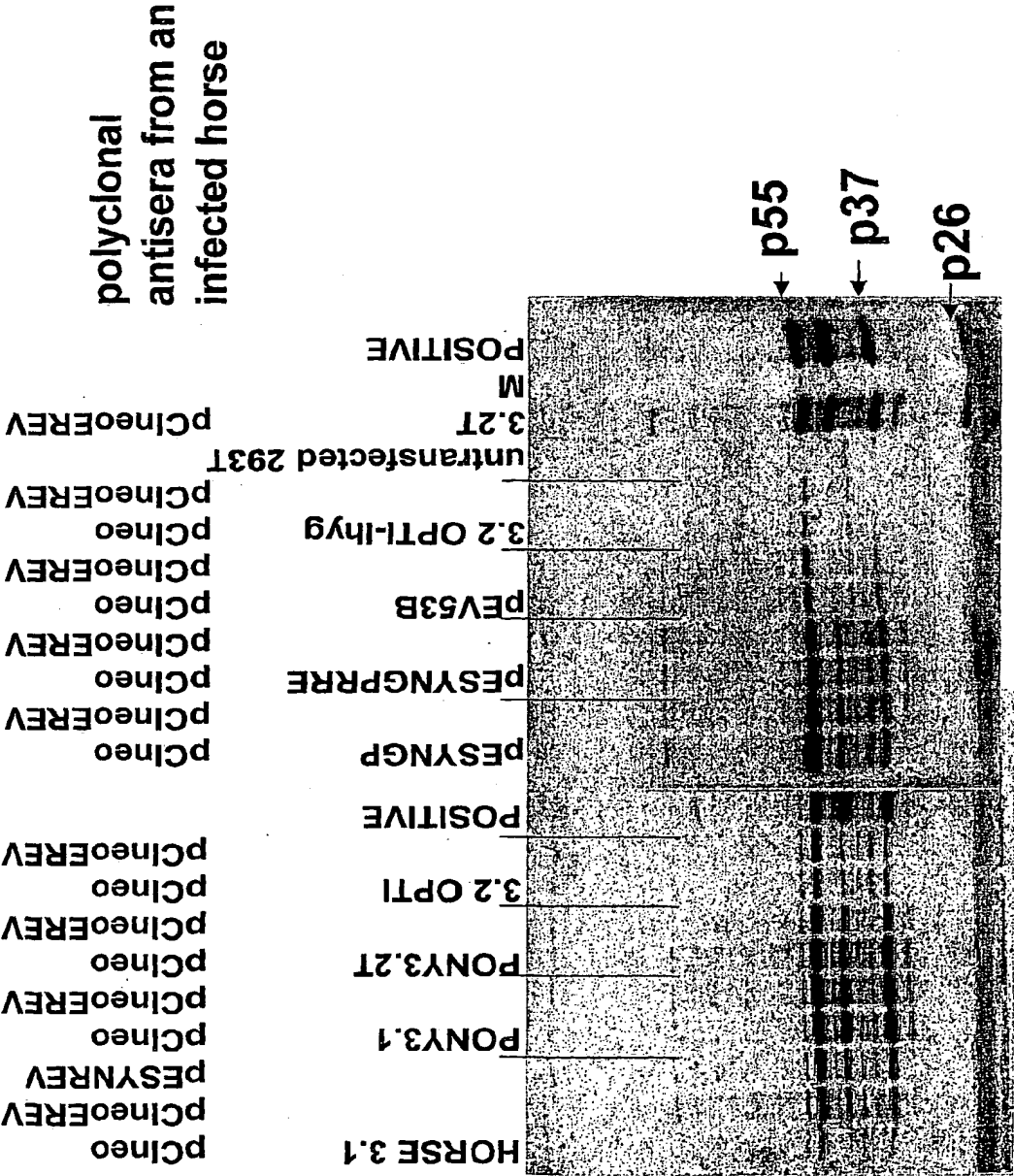
Vector genome	Gag length (nt)	SD mutation	pSYNGP	pGP-RRE3	pGP-RRE + Rev *
pH6nZ	625	No	$6.7 \pm 0.7 \times 10^5$	Not done	Not done
pH6.1nZ	625	No	$3.2 \pm 0.1 \times 10^4$	Not done	Not done
pH6.2nZ	527	No	$2.7 \pm 0.3 \times 10^4$	Not done	Not done
pHS1nZ	40	No	$2.1 \pm 0.4 \times 10^4$	$2.1 \pm 0.9 \times 10^3$	$1.4 \pm 0.3 \times 10^4$
pHS2nZ	260	No	$1.5 \pm 0.3 \times 10^4$	$4.2 \pm 0.6 \times 10^3$	$3.0 \pm 0.5 \times 10^4$
pHS3nZ	360	No	$1.3 \pm 0.3 \times 10^5$	$6.5 \pm 0.7 \times 10^3$	$1.7 \pm 0.4 \times 10^5$
pHS4nZ	625	No	$1.0 \pm 0.9 \times 10^4$	$1.2 \pm 0.7 \times 10^3$	$1.0 \pm 0.3 \times 10^4$
pHS5nZ	40	Yes	$2.0 \pm 0.5 \times 10^4$	$6.0 \pm 0.8 \times 10^2$	$2.3 \pm 0.5 \times 10^4$
pHS6nZ	260	Yes	$1.0 \pm 0.2 \times 10^4$	$8.1 \pm 0.6 \times 10^2$	$1.0 \pm 0.4 \times 10^4$
pHS7nZ	360	Yes	$1.9 \pm \times 10^4$	$2.4 \pm 0.9 \times 10^3$	$4.2 \pm 0.8 \times 10^4$
pHS8nZ	625	Yes	$8.0 \pm 1.0 \times 10^3$	$1.0 \pm 0.5 \times 10^3$	$4.0 \pm 0.9 \times 10^3$

Figure 24

Vector genome	pSYNGP	PSYNGP + Rev *
pH6nZ	6.5×10^5	6.9×10^5
pH6.1nZ	1.5×10^4	1.7×10^4
pH6.1nZR	2.3×10^3	1.6×10^5
pHS1nZ	8.5×10^3	8.4×10^3
pHS1nZR	8.1×10^3	8.8×10^4
pHS3nZ	1.2×10^5	1.4×10^5
pHS3nZR	2.5×10^3	4.8×10^5
pHS7nZ	7.3×10^3	7.0×10^3
pHS7nZR	3.4×10^3	5.8×10^3

Western blot of 293T extracts

Figure 26



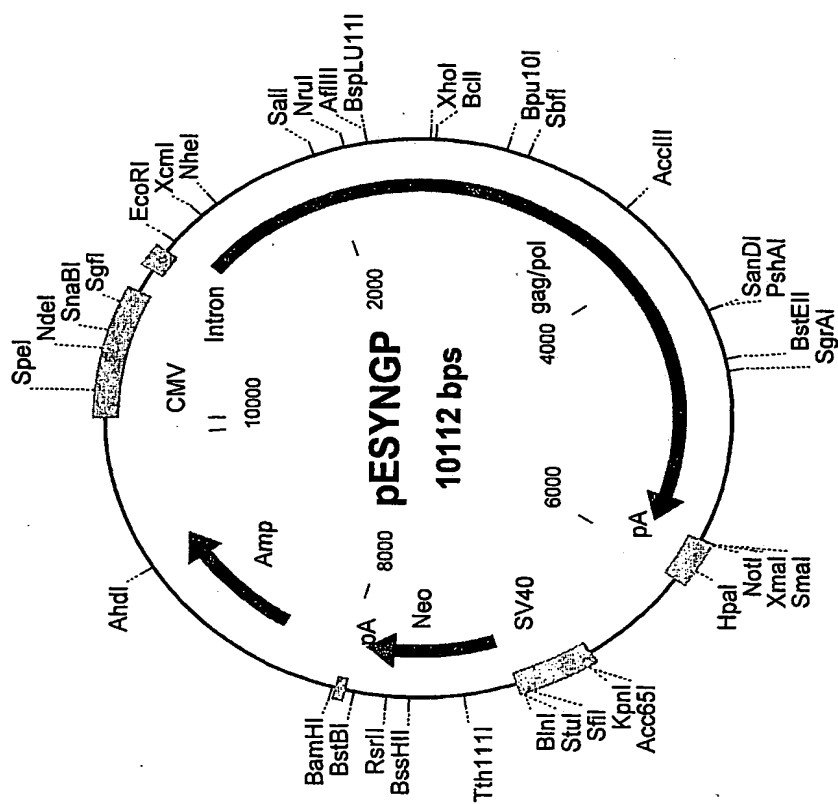
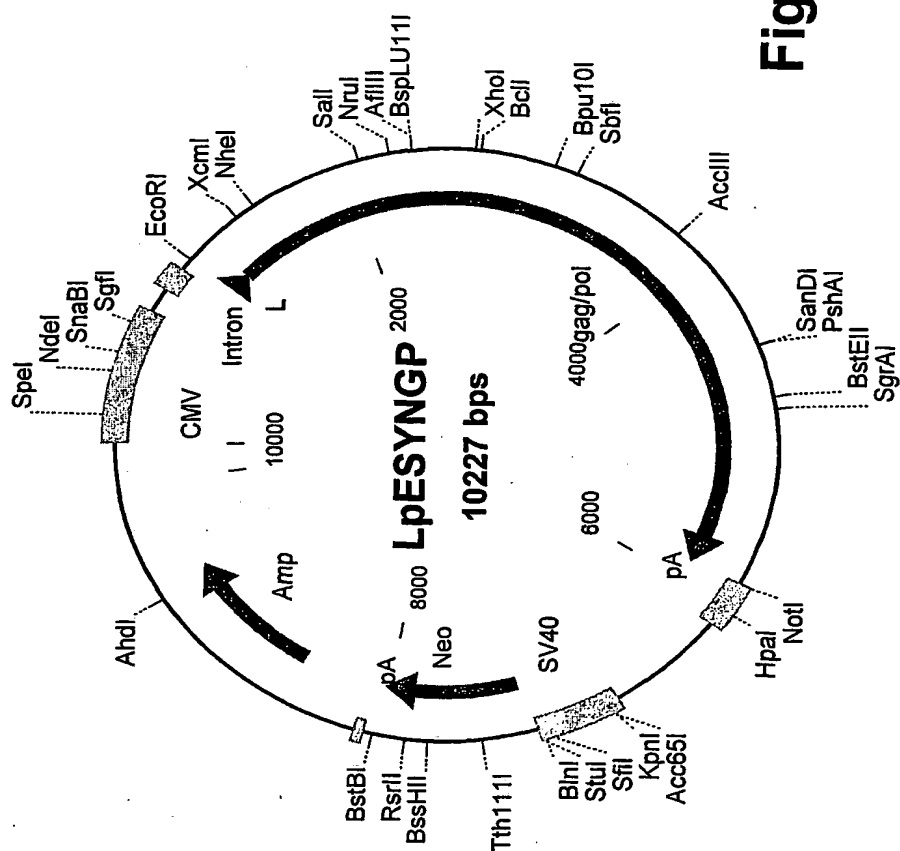


Figure 27

Figure 28



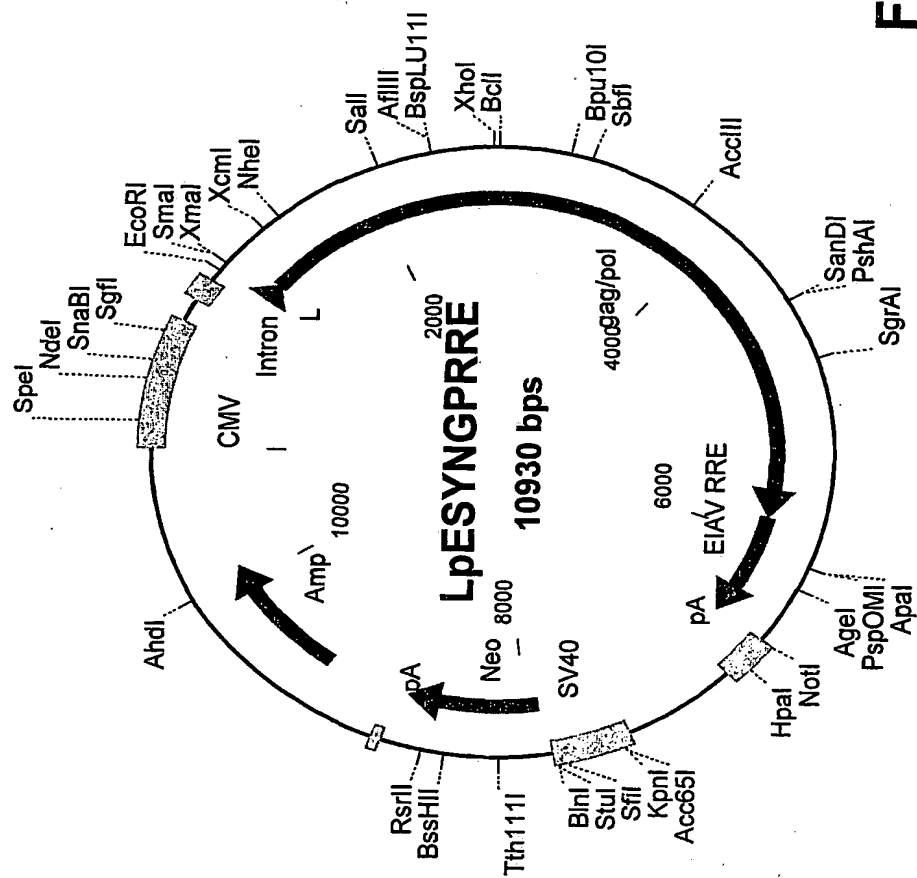


Figure 29

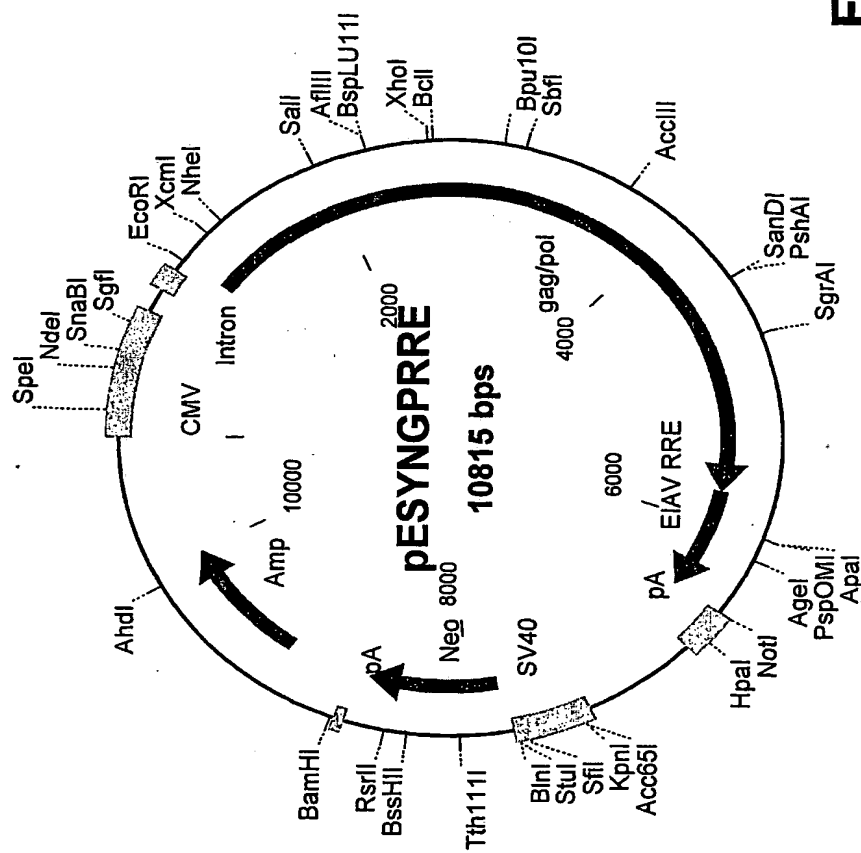


Figure 30

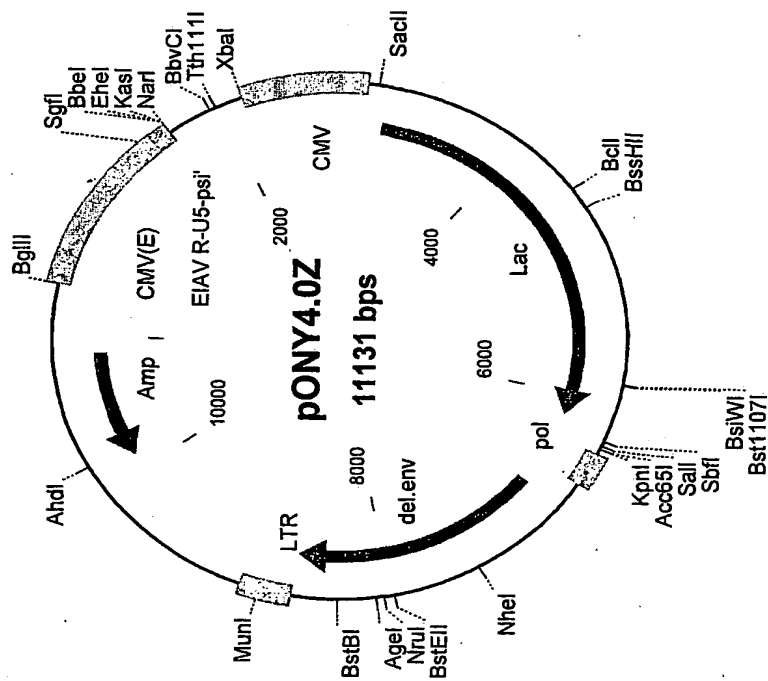
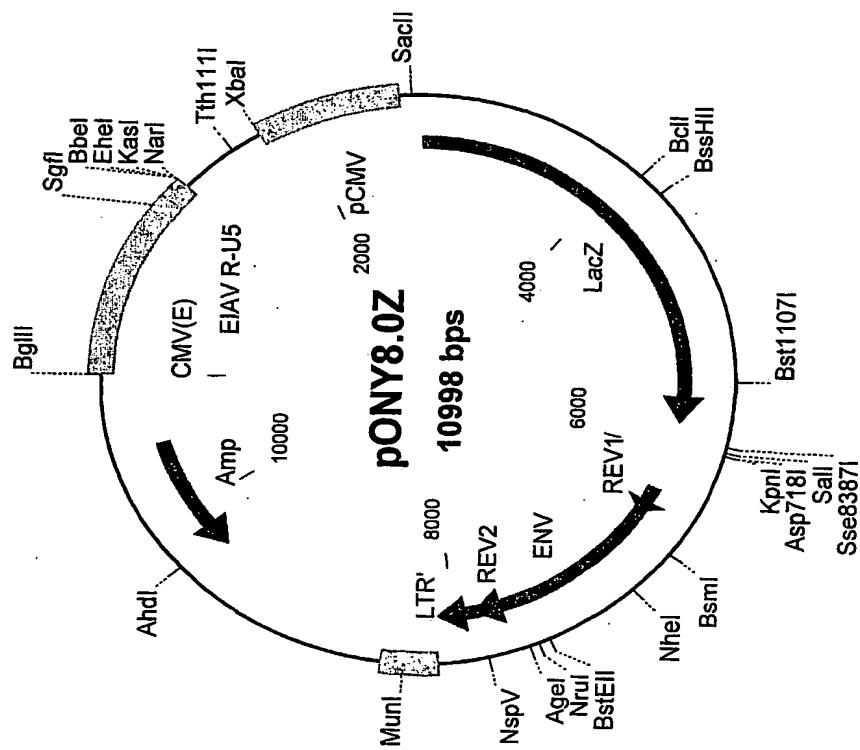


Figure 31

Figure 32



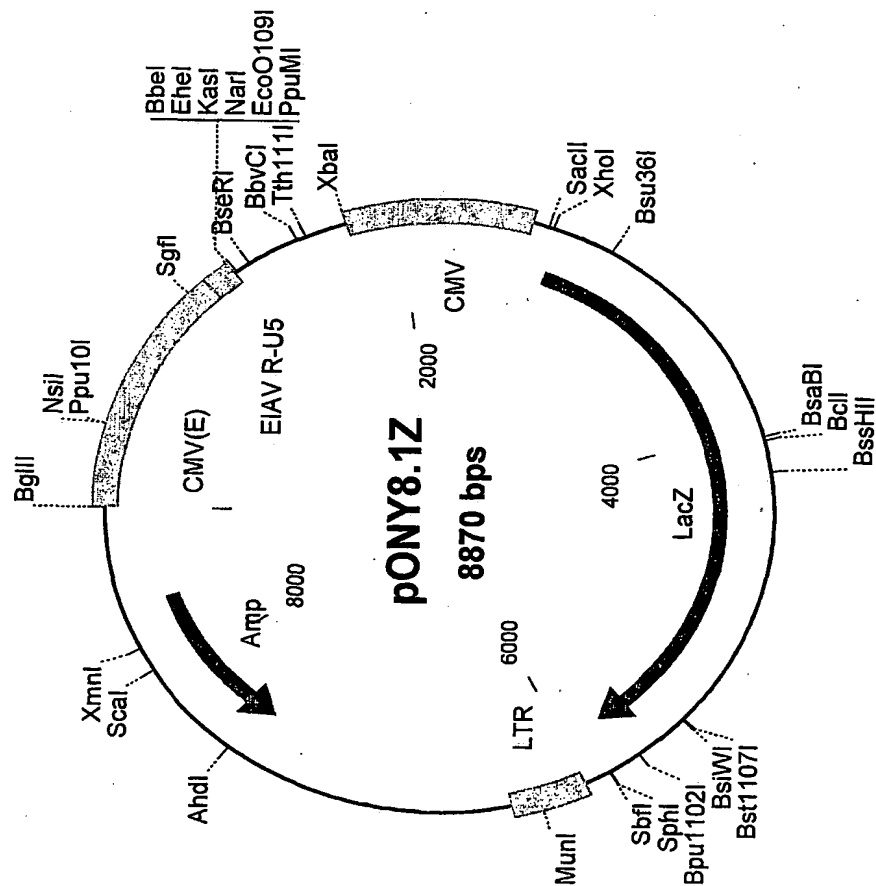


Figure 33

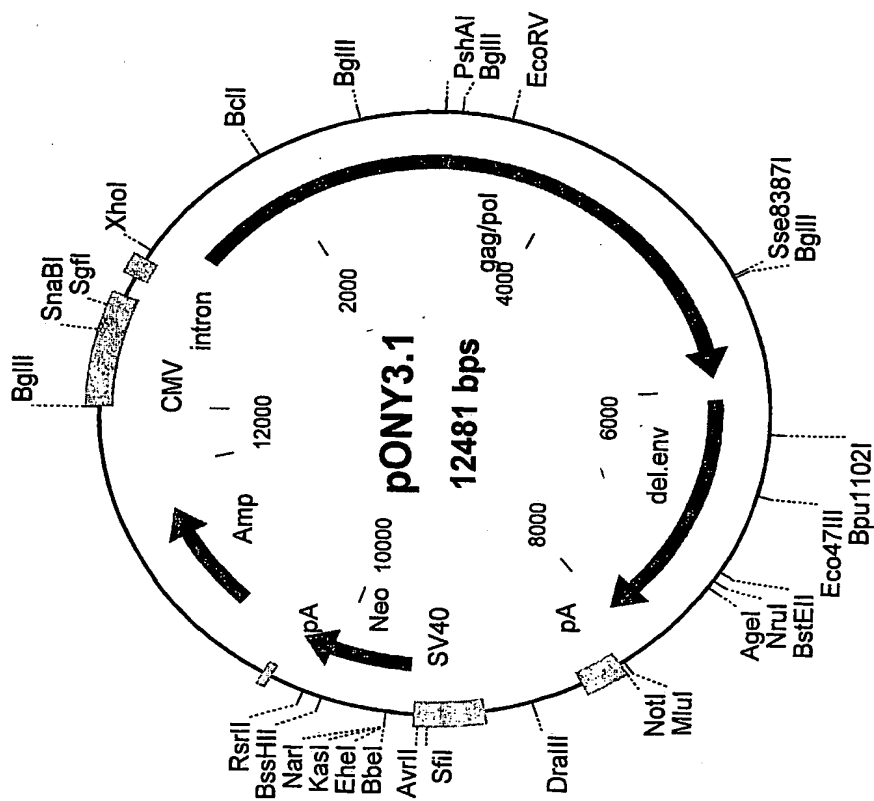


Figure 34

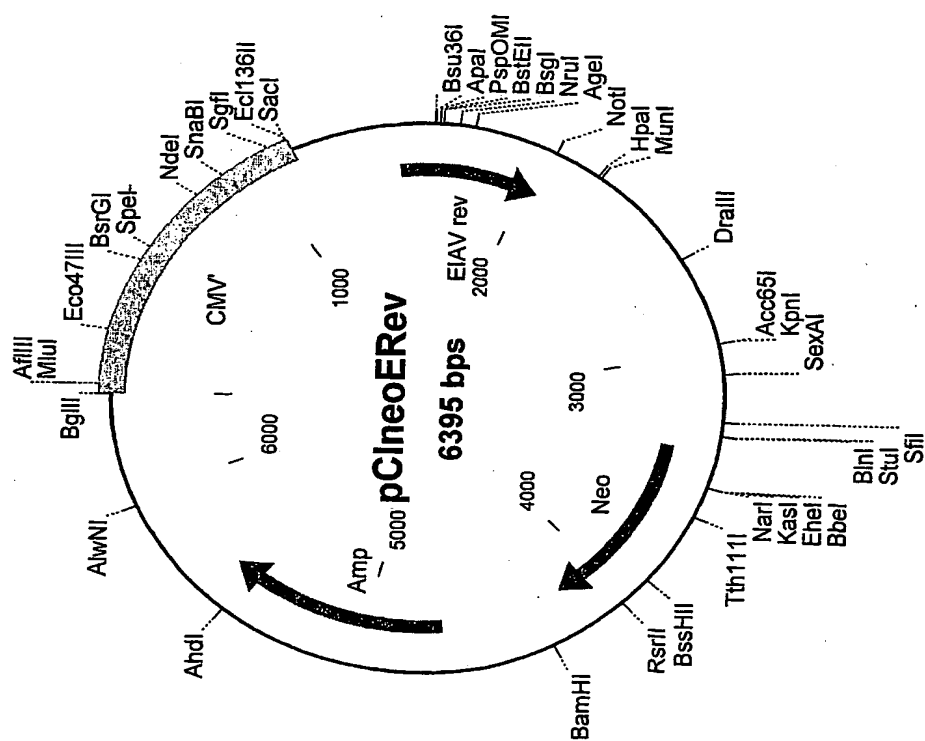


Figure 35

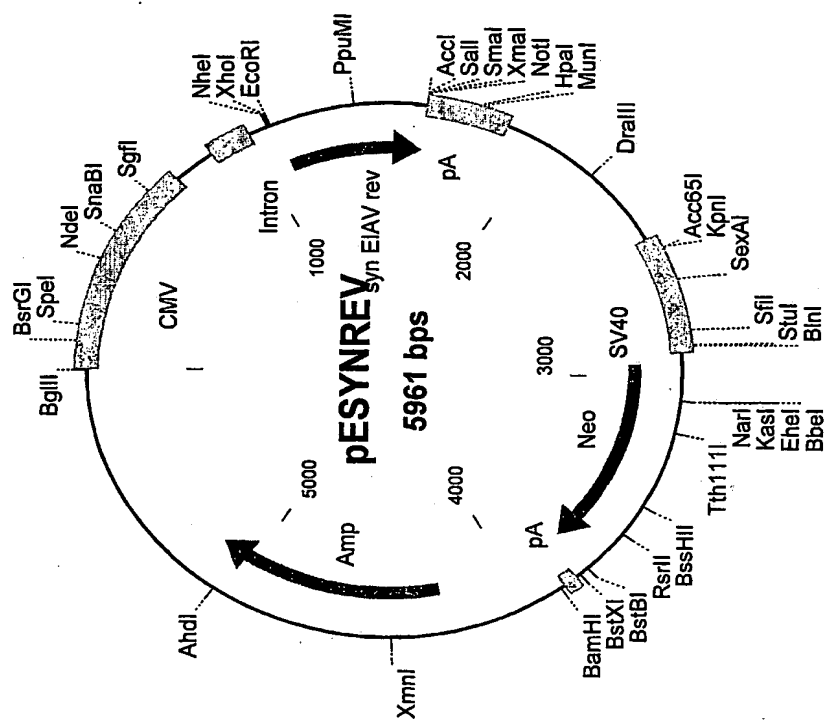


Figure 36

FIGURE 37

	Genome	Other	TU/ml 1	TU/ml 2	Mean
GAG-POL					
pONY3.1	pONY4.0Z	none	1.84E+06	2.50E+06	2.01E+06
pESYNGP	pONY4.0Z	none	9.00E+05	1.04E+06	9.67E+05
pHORSE3.1	pONY4.0Z	pCIneo	8.80E+04	1.26E+05	1.07E+05
pHORSE3.1	pONY4.0Z	pCIneoREV	1.48E+05	1.42E+05	1.45E+05
pONY3.1	pONY8.0Z	none	1.66E+06	1.48E+06	1.61E+06
pESYNGP	pONY8.0Z	none	2.06E+05	1.10E+05	1.91E+05
pESYNGP	pONY8.0Z	pCIneo	4.00E+04	3.40E+04	2.93E+04
pESYNGP	pONY8.0Z	pCIneoREV	6.40E+04	8.20E+04	6.47E+04
pHORSE3.1	pONY8.0Z	pCIneo	2.00E+03	2.00E+03	2.00E+03
pHORSE3.1	pONY8.0Z	pCIneoREV	1.80E+05	2.48E+05	2.14E+05

Figure 38

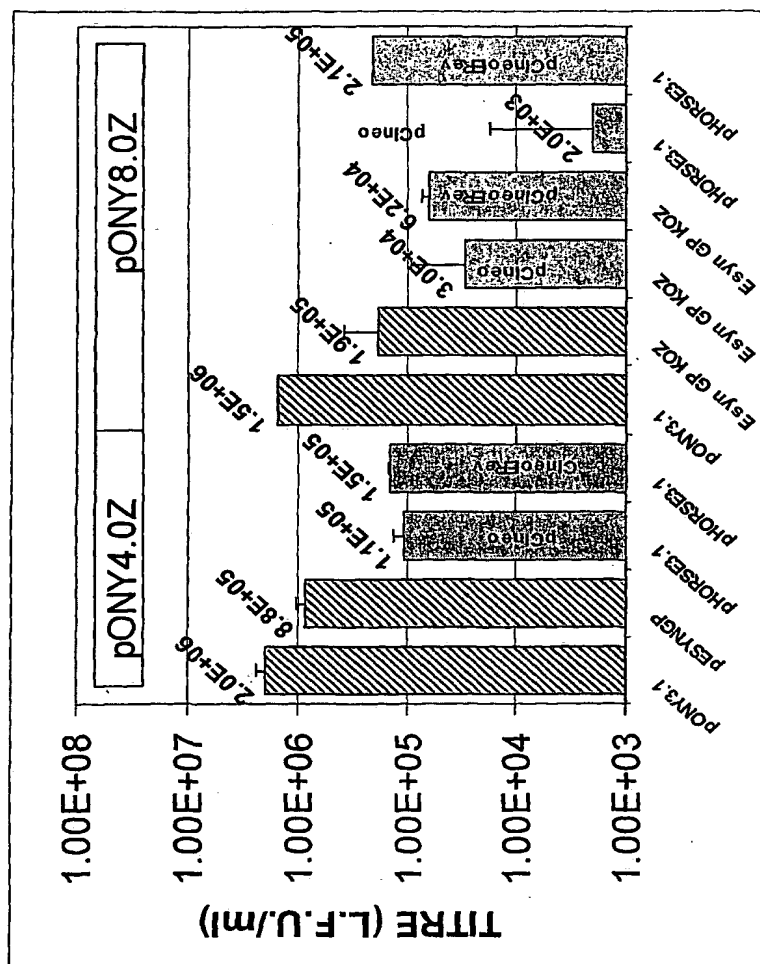


FIGURE 39

GAG/POL	OTHER	RT Activity
pONY3.1	pCIneo	87557.9
pONY3.1	pCIneoREV	147061.5
pESYNGP	pCIneo	45701.3
pESYNGP	pCIneoREV	63610.2
L pESYNGP	pCIneo	91729.9
L pESYNGP	pCIneoREV	88487.9
L pESYNGPRRE	pCIneo	71873.4
L pESYNGPRRE	pCIneoREV	101010.7
pESYNGPRRE	pCIneo	32823.1
pESYNGPRRE	pCIneoREV	44863.4
Untransfected 293T cells	NA	1024.6
No cells	NA	1330.6

FIGURE 40

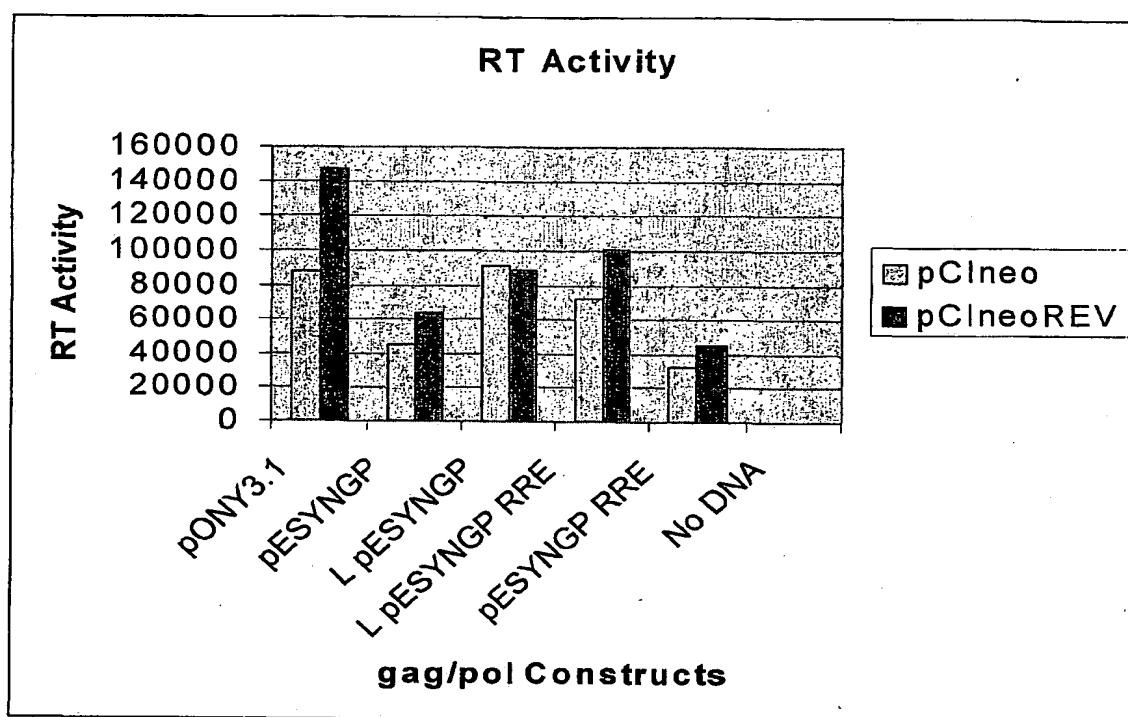


FIGURE 41

VECTOR	GAG/POL	OTHER	titre/ml 1	titre/ml 2	mean
pONY8.0Z	pONY3.1	pCIneo	3.20E+05	2.68E+05	2.94E+05
pONY8.0Z	pONY3.1	pCIneoREV	3.00E+05	5.00E+05	4.00E+05
pONY8.0Z	pESYNGP	pCIneo	4.00E+04	5.60E+04	4.80E+04
pONY8.0Z	pESYNGP	pCIneoREV	2.22E+05	2.40E+05	2.31E+05
pONY8.0Z	L pESYNGP	pCIneo	6.00E+04	6.60E+04	6.30E+04
pONY8.0Z	L pESYNGP	pCIneoREV	2.80E+05	2.70E+05	2.75E+05
pONY8.0Z	L pESYNGPRRE	pCIneo	9.20E+04	6.60E+04	7.90E+04
pONY8.0Z	L pESYNGPRRE	pCIneoREV	3.68E+05	2.82E+05	3.25E+05
pONY8.0Z	pESYNGPRRE	pCIneo	3.20E+04	1.40E+04	2.30E+04
pONY8.0Z	pESYNGPRRE	pCIneoREV	1.20E+05	1.40E+05	1.30E+05

FIGURE 42

GAG/POL	VECTOR	OTHER	titre/ml 1
pONY3.1	pONY8.0Z	pCIneo	1.2E+06
pONY3.1	pONY8.0Z	pCIneoERev	1.0E+06
pONY3.1	pONY8.1Z	pCIneo	1.0E+05
pONY3.1	pONY8.1Z	pCIneoERev	8.0E+05
pESYNGP	pONY8.0Z	pCIneo	2.3E+05
pESYNGP	pONY8.0Z	pCIneoRev	1.0E+05
pESYNGP	pONY8.1Z	pCIneo	1.0E+04
pESYNGP	pONY8.1Z	pCIneoRev	5.1E+04

Figure 43

WO 01/79518

PCT/GB01/01784

Comparison of PONY3.1 (nt1246-1606) (TOP) and PONY3.2opti (nt366-726) (BOTTOM)

```
atgggagaccctttgacatggagcaagcgctcaagaagttagagaagtgacggtacaa
.....C..t..cc.C..C...tc...a..C..g..a..ac.g..a..a..C..C..t..g

gggtctcagaaaattaactactggtaactgtaattgggcgctaagtctagtagacttattt
..tagc..a..gc.t..c..a..c..t..c..C...at.gtcc..g..g..tc.t..c

catgataccaactttgtaaaagaaaggactggcagctgagggatgtcattccattgctg
..C..C..t..t..C..t..g..g..a..t.....a..c..a..c..g..C..cc.ct..

gaagatgtaactcagacgctgtcaggacaagaagagagccctttgaaagaacatggtgg
..g..C..g..C..a..at...t..g..g..gc.C..a..t..C..gc.C..C.....

gcaatttctgctgtaaagatggcctccagattaataatgtagtagatggaaggcatca
..C..cagc..a..C..a.....g..g..a..C..C..c..g..t..c..t..a..tagc

ttccagctcctaagagcgaatatatgaaagaagactgctaataaaaaagcagctgagccc
..t..a..g..cc.C..t..g..C..g..a..a..C..C..C..g..a..a..C..a..t

tctgaagaatatccaatcatgatag
agc..g..g..C.....
```

pIRES1hyg ESYNGP

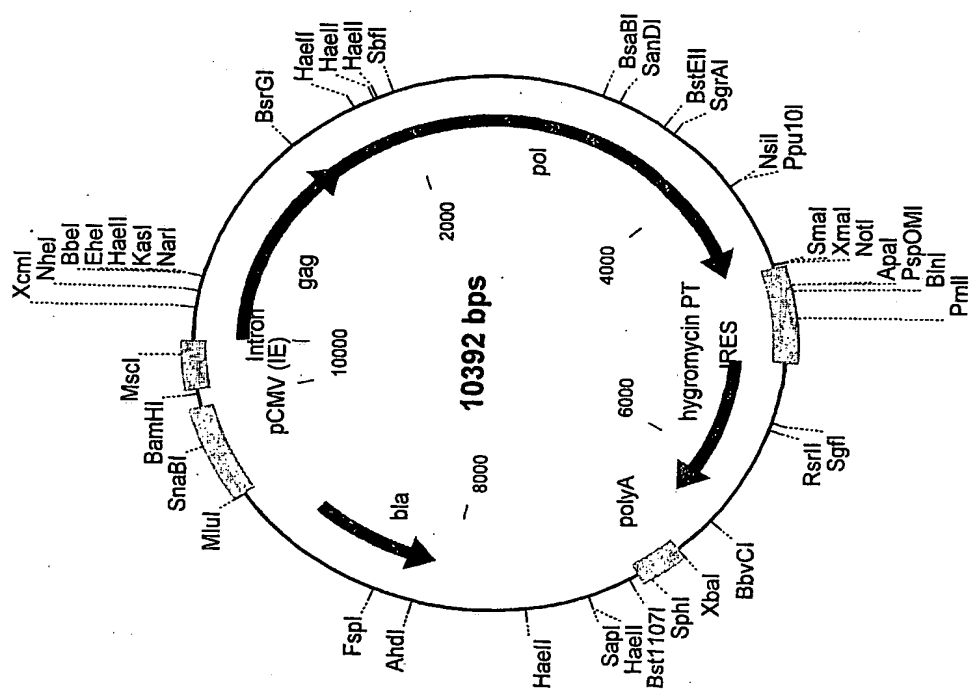


Figure 45

cell line	Ct value	$Ct_{\text{cell line}} - Ct_{\text{pONY8G transient}}$
Q3.29 (3 x 10 ⁵ cells)	17.4	-2.9
8Z.20(24h after induction)	14.35	-6.08
8Z.20(48h after induction)	14.81	-5.62
8Z.20(72h after induction)	16.19	-4.24
pONY8G	20.43	-

Figure 46

mass of plasmid transfected (μ g)/10cm diameter dish					titre on D17 cells (LacZ forming units/ml)			
transfection number	PESYNGP	PONY8.3G	PONY8.0Z	PESYNREV	PRV67	pCIneo	1 st harvest	2 nd harvest
1	16 μ g	16			8		1.4×10^5	1.6×10^5
2		16			8	16	1.4×10^5	1.6×10^5
3		16			8		1.4×10^5	2.6×10^5
4		32			8		2.8×10^6	1.1×10^6
5			16	8	8		2.4×10^5	2.8×10^5
6			32	8	8		4.5×10^6	1.1×10^6

Figure 47

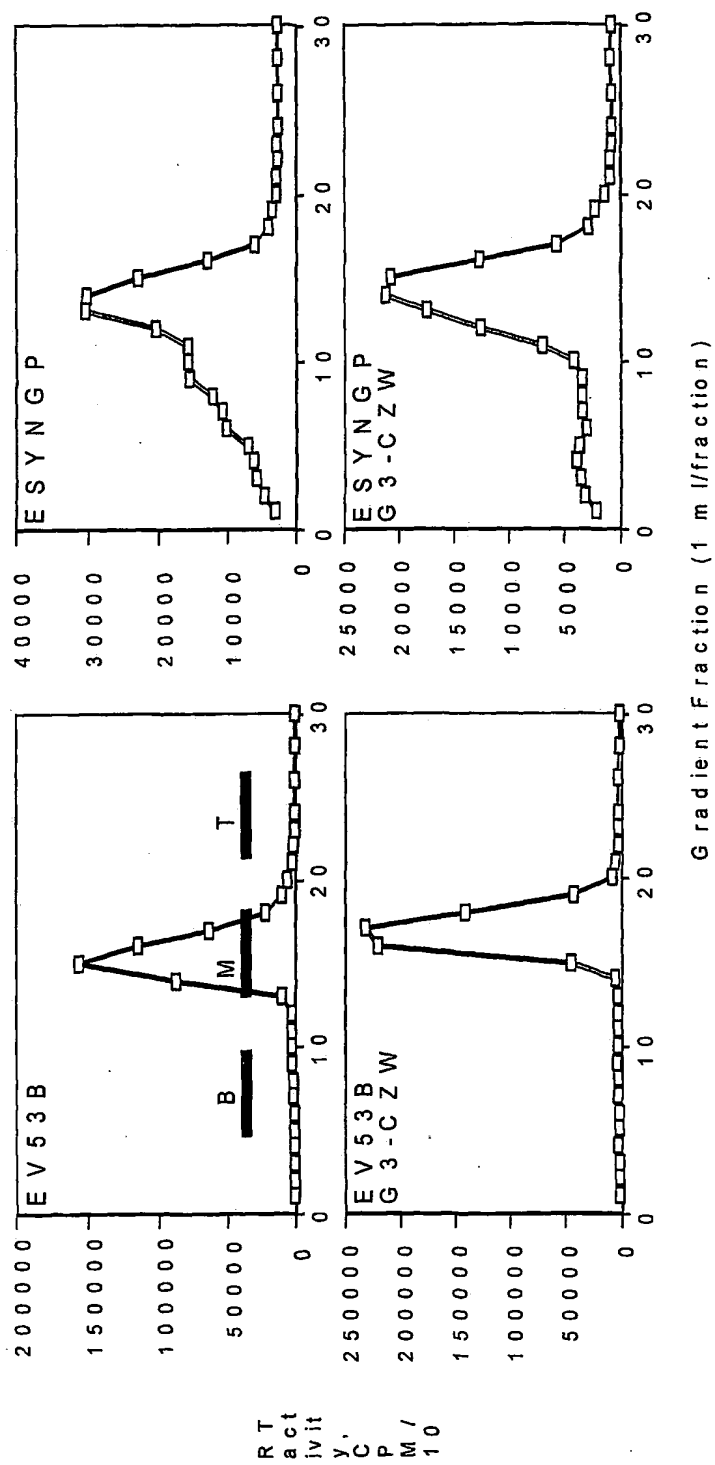


Figure 48

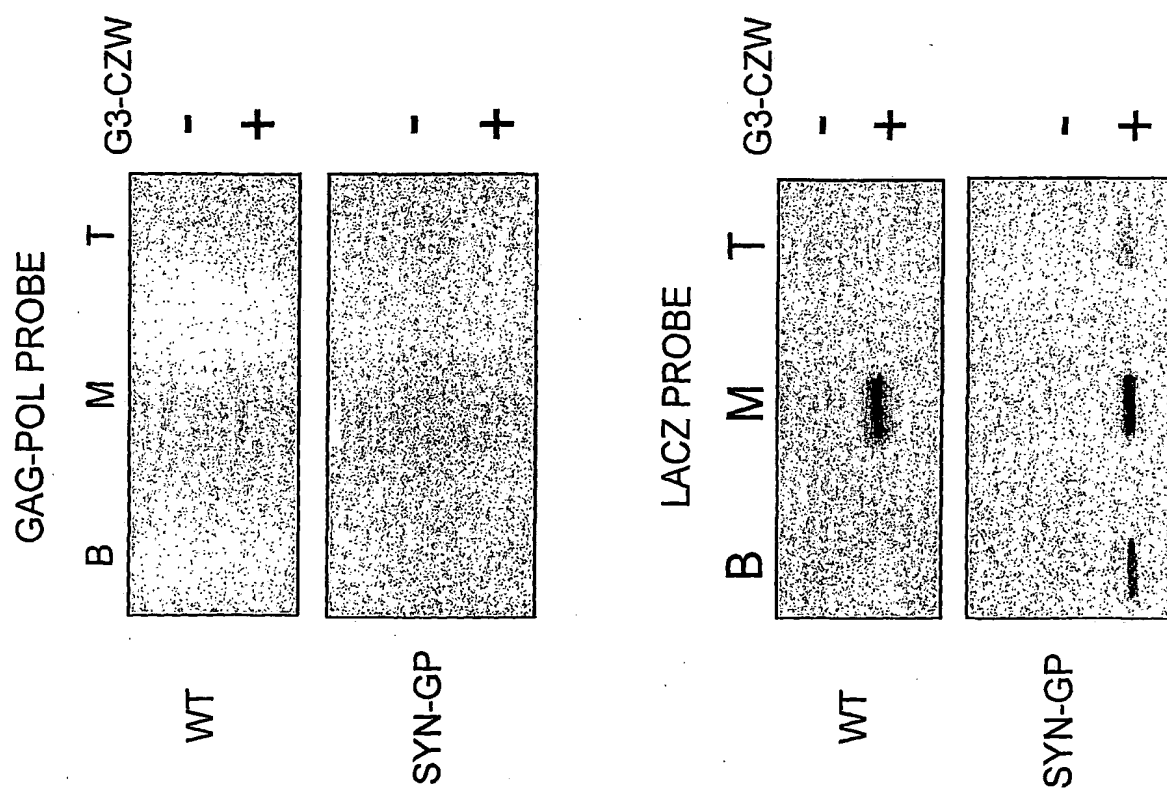


Figure 49

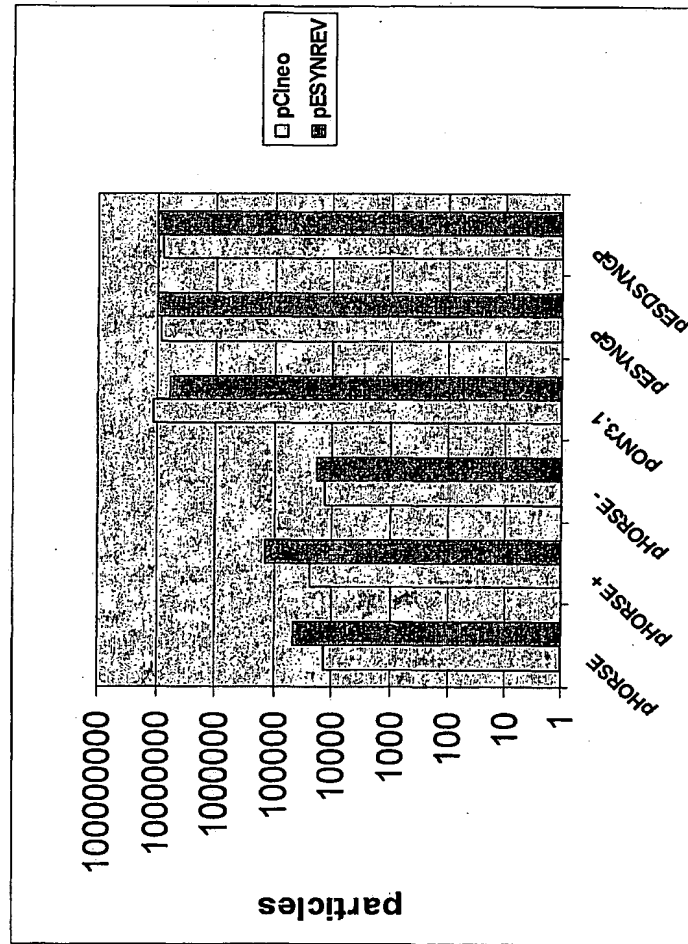


Figure 50

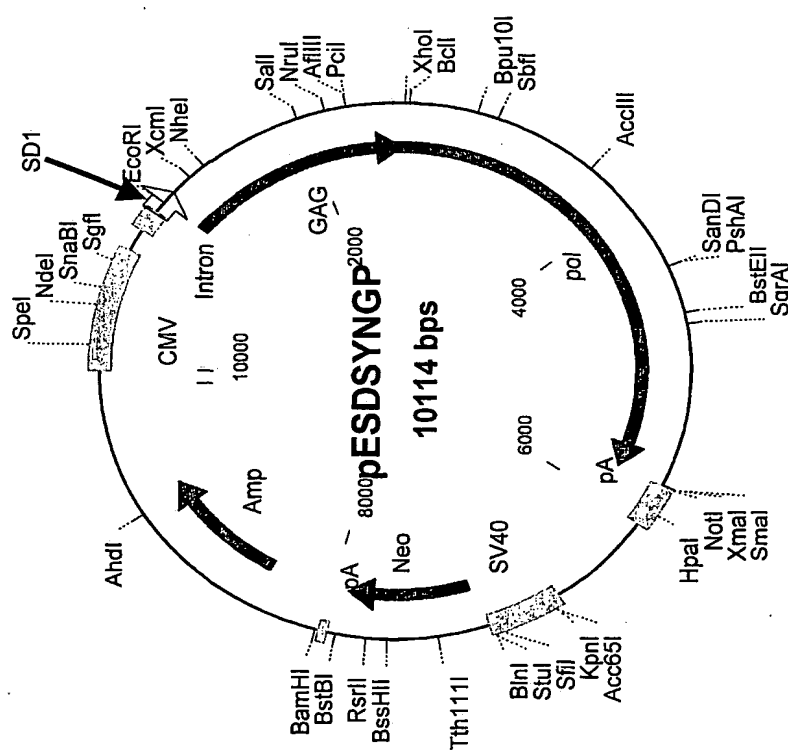
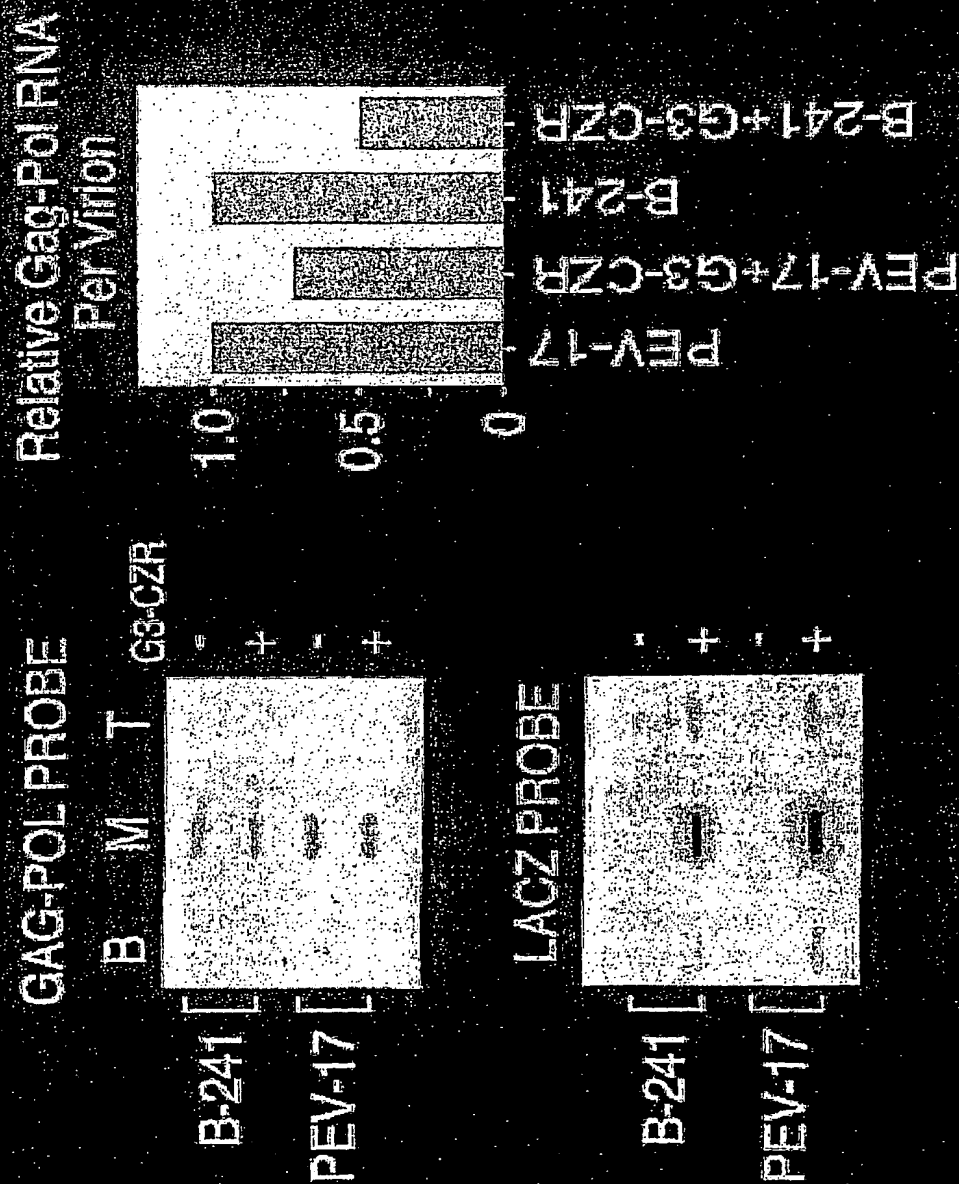


FIGURE 51



SEQUENCE LISTING PART OF THE DESCRIPTION

SEQ. ID. NO. 1 - Wild type gagpol sequence for strain HXB2 (accession no. K03455)

ATGGGTGCGA GAGCGTCAGT ATTAAGCGGG GGAGAATTAG ATCGATGGGA AAAAATTCGG 60
 TTAAGGCCAG GGGGAAAGAA AAAATATAAA TTA AACATA TAGTATGGGC AAGCAGGGAG 120
 CTAGAACGAT TCGCAGTTAA TCCTGGCCTG TTAGAAACAT CAGAAGGCTG TAGACAAATA 180
 CTGGGACAGC TACAACCATC CCTTCAGACA GGATCAGAAG AACTTAGATC ATTATATAAT 240
 ACAGTAGCAA CCCTCTATTG TGTGCATCAA AGGATAGAGA TAAAAGACAC CAAGGAAGCT 300
 TTAGACAAGA TAGAGGAAGA GCAAAACAAA AGTAAGAAAA AAGCACAGCA AGCAGCAGCT 360
 GACACAGGAC ACAGCAATCA GGTCAGCCAA AATTACCCTA TAGTGCAGAA CATCCAGGGG 420
 CAAATGGTAC ATCAGGCCAT ATCACCTAGA ACTTTAAATG CATGGGTAAA AGTAGTAGAA 480
 GAGAAGGCTT TCAGCCAGA AGTGATACCC ATGTTTTTCAG CATTATCAGA AGGAGCCACC 540
 CCACAAGATT TAAACACCAT GCTAAACACA GTGGGGGGAC ATCAAGCAGC CATGCAAAATG 600
 TTAAGAGAGA CCATCAATGA GGAAGCTGCA GAATGGGATA GAGTGCATCC AGTGCATGCA 660
 GGGCCTATTG CACCAGGCCA GATGAGAGAA CCAAGGGGAA GTGACATAGC AGGAACTACT 720
 AGTACCCTTC AGGAACAAAT AGGATGGATG ACAAATAATC CACCTATCCC AGTAGGAGAA 780
 ATTTATAAAA GATGGATAAT CCTGGGATTA AATAAAATAG TAAGAATGTA TAGCCCTACC 840
 AGCATTCTGG ACATAAGACA AGGACCAAAG GAACCCTTTA GAGACTATGT AGACCGGTTT 900
 TATAAACTC TAAGAGCCGA GCAAGCTTCA CAGGAGGTAA AAAATTGGAT GACAGAAACC 960
 TTGTTGGTCC AAAATGCGAA CCCAGATTGT AAGACTATTT TAAAAGCATT GGGACCAGCG 1020
 GCTACACTAG AAGAAATGAT GACAGCATGT CAGGGAGTAG GAGGACCCGG CCATAAGGCA 1080
 AGAGTTTTGG CTGAAGCAAT GAGCCAAGTA ACAAATTCAG CTACCATAAT GATGCAGAGA 1140
 GGCAATTTTA GGAACCAAAG AAAGATTGTT AAGTGTTCAT ATTGTGGCAA AGAAGGGCAC 1200
 ACAGCCAGAA ATTGCAGGGC CCCTAGGAAA AAGGGCTGTT GGAAATGTGG AAAGGAAGGA 1260
 CACCAAATGA AAGATTGTAC TGAGAGACAG GCTAATTTTT TAGGGAAGAT CTGGCCTTCC 1320
 TACAAGGGAA GGCCAGGGAA TTTTCTTCAG AGCAGACCAG AGCCAACAGC CCCACCAGAA 1380
 GAGAGCTTCA GGTCTGGGGT AGAGACAACA ACTCCCCCTC AGAAGCAGGA GCCGATAGAC 1440
 AAGGAACTGT ATCCTTTAAC TTCCCTCAGG TCACTCTTTG GCAACGACCC CTGCTCACAA 1500
 TAAAGATAGG GGGGCAACTA AAGGAAGCTC TATTAGATAC AGGAGCAGAT GATACAGTAT 1560
 TAGAAGAAAT GAGTTTGCCA GGAAGATGGA AACCAGAAAT GATAGGGGGA ATTGGAGGTT 1620
 TTATCAAAGT AAGACAGTAT GATCAGATAC TCATAGAAAT CTGTGGACAT AAAGCTATAG 1680
 GTACAGTATT AGTAGGACCT ACACCTGTCA ACATAATTGG AAGAAATCTG TTGACTCAGA 1740
 TTGGTTGCAC TTAAATTTTT CCCATTAGCC CTATTGAGAC TGTACCAGTA AAATTAAGC 1800
 CAGGAATGGA TGGCCCAAAA GTTAAACAAT GGCCATTGAC AGAAGAAAAA ATAAAGCAT 1860
 TAGTAGAAAT TTGTACAGAG ATGGAAAAGG AAGGGAAAAA TTCAAAAATT GGGCCTGAAA 1920
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SEQ. ID. NO. 2 - pSYNGP

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SEQ. ID. NO. 3- Envelope Gene from HIV-1 MN (Genbank accession no. M17449)

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SEQ. I.D. NO. 4 - SYNgp-160mn - codon optimised env sequence

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SEQ ID No. 5 - pESYNGP

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SEQ ID No. 6 - LpESYNGP

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SEQ ID No. 8 - LpESYNGPRRE

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Seq ID No: 15 codon optimised HIV gag-pol

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Seq ID No: 16 codon optimised EIAV gag-pol

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SEQ ID NO:17

pIRES1hyg ESYNGP

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CGGC

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(54) Title: CODON OPTIMISATION FOR EXPRESSION IN RETROVIRUS PACKAGING CELLS

Codon usage in human genes (MH), wild type HIV-1 Gag-pol (WT) and the codon optimised HIV-1 Gag-pol (CO)

[illegible]**b**

(57) Abstract: A method of producing a replication defective retrovirus comprising transfecting a producer cell with the following: i) a retroviral genome; ii) a nucleotide sequence coding for retroviral gag and pol proteins; and iii) nucleotide sequences encoding other essential viral packaging components not encoded by the nucleotide sequence of (ii); characterised in that the nucleotide sequence coding for retroviral gag and pol proteins is codon optimised for expression in the producer cell.

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patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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A. CLASSIFICATION OF SUBJECT MATTER

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A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, BIOSIS, EPO-Internal, WPI Data

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INTERNATIONAL SEARCH REPORT

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